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(54) Title: XYLANASE VARIANTS

(57) Abstract: The present invention relates to a variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor and has an altered thermo-sensitivity as compared with the parent xylanase enzyme.

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XYLANASE VARIANTS

Field of the invention

The present invention relates to mutant xylanase enzymes having an altered sensitivity to xylanase inhibitors as well as altered thermo-sensitivity. The present invention also relates to the use of these mutant enzymes in processing plant materials.

Background to the invention

For many years, endo-β-1,4-xylanases (EC 3.2.1.8) (referred to herein as xylanases) have been used for the modification of complex carbohydrates derived from plant cell wall material. It is well known in the art that the functionality of different xylanases (derived from different microorganisms or plants) differs enormously, as a function of pH and temperature. Based on these characteristics, different xylanases have been chosen for different industrial applications. Xylanases selected for the baking industry tend to be mesophilic regarding thermostability and temperature optima. Thus, they have activity at dough processing temperatures (typically 20-40°C) and they are inactivated during baking. In the feed industry, xylanases with different temperature characteristics are preferred. Optimally, xylanases for use in the feed industry are thermostable to retain activity after heat treatment and pelleting. Furthermore, it has recently been demonstrated, that inhibitors of xylanases may influence the functionality and efficiency of xylanases. However, combining these characteristics in variant polypeptides in not reported, and may be used to obtain tailored and more efficient xylanases.

Summary of the invention

The present invention relates to more useful xylanases and methods for obtaining same.

The data presented herein show that xylanase inhibitors dictate the functionality and efficiency of the xylanases currently used in, for example, wheat based systems. Furthermore, it is also shown that changing the xylanases sensitivity towards xylanase inhibitors, can be combined with changed thermo-sensitivity of the xylanase. Such a combination makes it possible to tailor a xylanase with the right functionality and thermo-sensitivity

Furthermore, we have gone on to design and test a series of xylanases modified by site-directed mutagenesis to demonstrate that xylanases can be produced that have a reduced sensitivity to xylanase inhibitors present in plant material and changes thermo-sensitivity.

In this context, thermo-sensitivity means the xylanases' thermostability and/or temperature optimum. Both characteristics known to persons skilled in the art.

In particular, we have identified a number of residues in family 11 xylanases which influence the degree of inhibition of the xylanase and a number of residues which influence the thermo-sensitivity.

Thus, it will be possible to produce variant xylanases having a modified (for example reduced) sensitivity to xylanase inhibitors and hence altered functionality and a modified thermo-sensitivity.

Altering the functionality, for example thermo-sensitivity and inhibitor sensitivity of a xylanase, will, for example, allow a reduction in the amount of xylanase required in a number of applications such as animal feed, starch production, bakery, flour separation (wetmilling), and paper and pulp production. In addition or alternatively, altering the functionality of a xylanase may make it possible to ensure that xylanase activity is inactivated at moderate, desirable temperatures, without using costly and often highly detrimental temperature elevations in the material processed.

The present invention provides a method of altering the sensitivity of a xylanase polypeptide to an inhibitor and of altering the thermo-sensitivity of said xylanase polypeptide, which method comprises modifying, preferably modifying one or more amino acid residues of, a parent xylanase polypeptide to provide a variant xylanase polypeptide, and testing the sensitivity of said variant xylanase polypeptide to a xylanase inhibitor compared with the parent xylanase enzyme and testing the thermosensitivity of said variant xylanase polypeptide compared with the parent xylanase enzyme and, optionally, selecting a variant xylanase polypeptide having an altered sensitivity to a xylanase inhibitor and an altered thermo-sensitivity compared with the parent xylanase enzyme.

The present invention further provides a variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor compared with a parent enzyme and has an altered thermo-sensitivity as compared with a parent enzyme.

The term "parent enzyme" as used herein means an xylanase enzyme from which the variant xylanase enzyme is derived or derivable. With respect to the term "derivable", the variant need not necessarily be derived from the parent enzyme. Instead, the variant could be prepared, for example, by use of recombinant DNA techniques that utilise nucleotide sequence(s) encoding said variant xylanase sequence - i.e. here the nucleotide sequence(s) are similar to mutated nucleotide sequence(s) but they are not prepared by mutation of the parent nucleotide sequence(s). The variant may even be prepared by chemically modifying a parent enzyme. For some embodiments the parent enzyme is the wild type enzyme. The term "wild type" is a term of art understood by skilled persons and includes a phenotype that is characteristic of most of the members of a species occurring naturally and contrasting with the phenotype of a mutant. Thus, in the present context, the wild type enzyme may be a form of the enzyme naturally found in most members of the relevant species. Generally, the relevant wild type enzyme in relation to the variant polypeptides of the invention is the most closely

related corresponding wild type enzyme in terms of sequence homology. For example, for the particular mutant xylanases described in the examples, the corresponding wild type enzyme is the wild type *Bacillus subtilis* xylanase A, more specifically the wild type *B. subtilis* xylanase A published by Paice *et al.*, 1986 and shown as SEQ ID No. 1. However, where a particular wild type sequence has been used as the basis for producing a variant polypeptide of the invention, this will be the corresponding wild type sequence regardless of the existence of another wild type sequence that is more closely related in terms of amino acid sequence homology. The parent may even be a naturally occurring mutant of the wild type. In a highly preferred aspect the polypeptide of the present invention is prepared by mutating (e.g. by use of biological and/or chemical techniques) a parent enzyme. For some embodiments, preferably the variant polypeptide is derived from a family 11 xylanase.

By way of example, the *B. subtilis* amino acid sequence for xylanase A is shown as SEQ ID No. 1. With respect to this sequence, the amino acid residues giving altered xylanase inhibitor sensitivity may be one or more of:

D11, G12, G13, I15, N17, N29, S31, N32, G34, Y113, N114, D119, G120, D121, R122, T123, T124 and Q175

In a further aspect, with respect to the *B. subtilis* amino acid sequence of xylanse A as shown in SEQ ID No. 1, the amino acid residues giving altered xylanase inhibitor sensitivity may be one or more of:

D11, G12, G13, G34, D121, R122 and T124

The mutations giving altered thermo-sensitivity may be the mutations mentioned below or combinations thereof in respect of the *B. subtilis* amino acid sequence for xylanase A as shown in SEQ ID No. 1:

S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R.

By choosing an appropriate combination of mutations, for example from the lists above, a xylanase with a desired inhibitor sensitivity and thermo-sensitivity can be obtained. Thus, the inhibitor sensitivity and thermo-sensitivity of the xylanase can be individually controlled.

Thus, preferably a variant xylanase polypeptide, or fragment thereof having xylanase activity according to the present invention has an amino acid modification at any one or more of the amino acid residues: 2, 8, 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 113, 114, 119, 120, 121, 122, 123, 124, 126, 128, 142, 143, 147, 154, 156, 163, 169, 175 and 179, or combinations thereof, based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

Preferably a variant xylanase polypeptide, or fragment thereof having xylanase activity according to the present invention has at least one amino acid modification at any one or more of the amino acid residues 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124, and 175 together with at least one amino acid modification to any one or more of the amino acid residues 2, 8, 15, 22, 29, 31, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 121, 123, 126, 128, 142, 143, 147, 154, 156, 163, 169 and 179 based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

Suitably, a variant xylanase polypeptide, or fragment thereof having xylanase activity according to the present invention may have an amino acid modification at any one or more of amino acid residues: 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 113, 114, 119, 120, 121, 123, 124, 126, 128, 142, 143, 147, 154, 156, 163, 169 and 179, or combinations thereof, based on the amino acid

numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

Suitably a variant xylanase polypeptide, or fragment thereof having xylanase activity according to the present invention may have at least one amino acid modification at any one or more of the amino acid residues 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 123 and 124 together with at least one amino acid modification to any one or more of the amino acid residues 15, 22, 29, 31, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 121, 123, 126, 128, 142, 143, 147, 154, 156, 163, 169 and 179 based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

By way of example only, suitable combinations of mutations may include:
D11F with one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R;

D11F and G13F, with additionally one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R;

D11F and G34D, with additionally one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R; or

D11F and R122D, with additionally one or more of S2A, N8Y, I15M, S22P, N29T, S31Q, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N, K99N, T111M, D121T, T123Q, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R.

The present invention further relates to a variant xylanase obtainable by, preferably obtained by, either directly or indirectly the method according to the present invention.

In a further embodiment, the present invention relates to a further variant xylanase prepared from a variant xylanase obtainable by, preferably obtained by, carrying out the method of the present invention.

Preferably, the inhibitor is an inhibitor found naturally in plant tissues. Suitably the inhibitor may be a *Triticum aestivum* xylanse-inhibitor (TAXI). Preferably the sensitivity of the variant xylanase enzyme to the inhibitor is reduced as compared to the parent xylanase enzyme.

Preferably, the thermostability of the variant xylanase enzyme is increased.

Preferably, the thermostability of the variant xylanase enzyme is increased by at least 5%, more preferably by at least 10%, even more preferably by at least 15%, even more preferably by at least 20%, even more preferably by at least 30%, even more preferably by at least 40%, even more preferably by at least 50% relative to the parent enzyme when determined using the "Thermostability assay" detailed hereinbelow.

Preferably, the thermosensitivity of the variant xylanase is decreased.

Preferably, the thermosensitivity of the variant xylanase enzyme is decreased by at least 5%, more preferably by at least 10%, even more preferably by at least 15%, even more preferably by at least 20%, even more preferably by at least 30%, even more preferably by at least 40%, even more preferably by at least 50% relative to the parent enzyme.

Preferably, the variant xylanases have a reduced sensitivity to xylanase inhibitors.

Preferably, the sensitivity of the variant xylanase enzyme to a xylanase inhibitor is reduced by at least 5%, more preferably by at least 10%, even more preferably by at least 15%, even more preferably by at least 20%, even more preferably by at least 30%, even more preferably by at least 40%, even more preferably by at least 50% relative to the parent enzyme when determined using the "Xylanase inhibitor assay" detailed hereinbelow.

The present invention also provides a nucleic acid molecule (a nucleotide sequence) encoding a polypeptide of the invention. Also provided is a vector comprising a nucleic acid of the invention, optionally operably linked to a regulatory sequence capable of directing expression of said nucleic acid in a suitable host cell. A host cell comprising a nucleic acid or a vector of the invention is also provided.

In another aspect the present invention provides a method of making a polypeptide of the invention comprising transforming a host cell with a nucleic acid encoding said polypeptide, culturing the transformed cell and expressing said polypeptide.

Our results show that these variant polypeptides have improved properties that make them suitable for a variety of applications, such as bakery, animal feed, starch production, flour separation (wetmilling) and, paper and pulp production.

Accordingly, the present invention also provides the use of a variant polypeptide of the invention in a method of modifying plant materials.

Also provided is the use of a variant polypeptide of the invention in baking. The invention further provides the use of a variant polypeptide of the invention in processing cereals, starch production and animal feed and the use of a variant polypeptide of the invention in processing wood, for example in enhancing the bleaching of wood pulp.

The invention further provides a dough that is obtainable by, preferably obtained by, the use of the variant polypeptide and baked products that are obtained by baking such a dough, and noodle and pasta products prepared in accordance with the invention.

Accordingly, the invention further relates to a method of preparing a flour dough, said method comprising adding to the dough components a variant xylanase in accordance with the present invention.

In a further aspect, there is provided a dough improving composition comprising a variant xylanase according to the present invention.

In the same way that variant xylanases may be used in a variety of processing applications, xylanase inhibitors may be used in a variety of processing applications such as bakery, wood pulp processing and cereal processing.

Detailed description of the invention

Although in general any molecular techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc.

A. Variant xylanase polypeptides

Xylanase enzymes have been reported from nearly 100 different organisms, including plants, fungi and bacteria. The xylanase enzymes are classified into several of the more than 40 families of glycosyl hydrolase enzymes. The glycosyl hydrolase enzymes, which include xylanases, mannanases, amylases, β-glucanases, cellulases and other carbohydrases, are classified based on such properties as the sequence of

amino acids, the three dimensional structure and the geometry of the catalytic site (Gilkes, et al., 1991, Microbiol. Reviews 55: 303-315).

Of particular interest for baking applications are the enzymes classified in Family 11. All of these are xylanases and are known as the "Family 11 xylanases". Some publications refer to these synonymously as the Family G xylanases, but the term "Family 11 xylanases" will be used herein to refer to both Family G and Family 11 xylanases.

Table A lists a number of known Family 11 xylanases. Most of them have a molecular mass of about 21,000 Da. Three of the Family 11 xylanases (*Clostridium stercorarium* XynA, *Streptomyces lividans* XynB, and *Thermomonospora fusca* XynA) have a higher molecular mass of 31,000 to 50,000 Da. However, these xylanases have a catalytic core sequence of about 21,000 Da similar to the other Family 11 xylanases. The amino acid sequences of the Family 11 xylanases (or, for the larger enzymes, the catalytic core) show a high degree of similarity, usually with more than 40 % identical amino acids in a proper amino acid alignment. The Family 11 xylanases, which are of bacterial, yeast, or fungal origin, share the same general molecular structure.

Figure 1 shows amino acid sequence alignment data in respect of 51 Family 11 xylanases.

TABLE A - Family 11 xylanases

Aspergillus niger Xyn A	*Aspergillus kawachii* Xyn C
Aspergillus tubigensis Xyn A	*Bacillus circulans* Xyn A
Bacillus pumilus Xyn A	*Bacillus subtilis* Xyn A
Cellulomonas fimi Xyn D	*Chainia* spp. Xyn
Clostridium acetobutylicum Xyn B	*Clostridium stercorarium* Xyn A

Fibrobacter succinogenes Xyn C	*Neocallimastix patriciarum* Xyn A
Nocardiopsis dassonvillei Xyn II	*Ruminococcus flavefaciens* Xyn A
Schizophyllum commune Xyn	*Streptomyces lividans* Xyn B
Streptomyces lividans Xyn C	*Streptomyces sp.* No. 36a Xyn
Streptomyces thermoviolaceus Xyn II	*Thermomonospora fusca* Xyn A
Trichoderma harzianum Xyn	*Trichoderma reesei* Xyn I
Trichoderma reesei Xyn II	*Trichoderma viride* Xyn

Variant xylanases of the invention

A variant xylanase polypeptide of the invention is typically obtained by modifying a xylanase polypeptide by substituting, deleting or adding one or more amino acid residues within the amino acid sequence of the xylanase polypeptide. Preferably the modification comprises one or more amino acid substitutions. Modification of polypeptide sequences can be carried out using standard techniques such as site directed mutagenesis. The modification may also occur by chemical techniques – such as chemical modification of one or more amino acid residues.

The starting sequence may be a wild type sequence or a non-naturally occurring sequence, for example a derivative that has already been subjected to protein engineering. The xylanase sequence to be modified may be from any source, for example a bacterial, fungal or plant source. Preferably the xylanase sequence to be modified is that of a Family 11 xylanase, more preferably a Family 11 xylanase selected from *Trichoderma reesei* xylanase I, *Trichoderma reesei* xylanase II, *Trichoderma harzianum* xylanase, *Trichoderma viride* xylanase, *Bacillus circulans* xylanase A, *Bacillus subtilis* xylanase A, *Bacillus stearothermophilus* xylanase A, *Aspergillus niger* xylanase A, *Aspergillus kawachii xylanase* C, *Aspergillus tubigensis* xylanase A, *Streptomyces lividans* xylanase B, and *Streptomyces lividans* xylanase C.

In a particularly preferred embodiment, the xylanase sequence to be modified is the *B. subtilis* xylanase sequence shown as SEQ ID No. 1 or a homologue thereof. Preferably said homologue has at least 40, 50, 60 or 80% homology over at least 50 or 100 amino acid residues as determined using the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387).

Specific modifications that are possible according to the present invention include one or more amino acid substitutions at positions 11, 12, 13, 15, 17, 29, 31, 32, 34, 113, 114, 119, 120, 121, 122, 123, 124 and 175 regarding inhibitor sensitivity and one or more substitutions at positions 2, 8, 15, 22, 29, 31, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 121, 123, 126, 128, 142, 143, 147, 154, 156, 163, 169 and 179 regarding thermo-sensitivity, or combinations thereof based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

Possible substitutions include one or more of D11→Y, D11→N, D11→F, D11→K, D11→S, D11→W, G12→F, G13→F, I15→K, N17→K, N17→Y, N17→D, N29→K, N29→Y, N29→D, S31→K, S31→Y, S31→D, N32→K, G34→D, G34→F, G34→T, Y113→A, Y113→D, Y113→K, N114→A, N114→D, N114→F, N114→K, D119→K, D119→Y, D119→N, G120→K, G120→D, G120→F, G120→Y, G120→N, D121→N, D121→K, D121→F, D121→A, R122→D, R122→F, R122→A, T123→K, T123→Y, T123→D, T124→K, T124→Y, T124→D, Q175→E, Q175→S and Q175→L regarding inhibitor sensitivity (with reference to the amino acid sequence of *B. subtilis* xylanase) or their equivalents in other homologous xylanase polypeptides and one or more substitutions of S2→A, N8→Y, I15→M, S22→P, N29→T, S31→Q, T44→V, F48→N, T50→V, V57→I, A59→E, N61→S, S74→N, P75→A, P90→A, T93→N, K99→N, T111→M, D121→T, T123→Q, T126→Q, Y128→F, A142→V, T143→S, T147→S, K154→R, H156→K, N163→S, M169→L, S179→R regarding thermo-sensitivity (with reference to the amino acid sequence of *B. subtilis* xylanase) or their equivalents in other homologous xylanase polypeptides and

combinations thereof. Further references to specific residues of the *B. subtilis* xylanase shown as SEQ ID No. 1 will also include their equivalents in other homologous xylanase polypeptides.

A combination of mutations may be carried out, for example mutations at two or more, three or more, four or more, five or more, ten or more, or fifteen or more of the above-mentioned residues.

In a further embodiment, the variant polypeptides of the invention may be purified and isolated naturally occurring mutant xylanases. Alternatively, mutant xylanases may be generated by subjecting organisms to mutagens and then screening for individuals comprising mutations in their xylanase genes. Naturally occurring mutants and mutants generated by random mutagenesis may be identified/screened using a variety of techniques such as PCR screening using suitable nucleic acid primers to amplify regions of xylanase genes and sequencing the resulting fragments.

Thus variant polypeptides of the invention include naturally occurring mutant xylanases (purified and isolated from the organisms in which they occur or obtained recombinantly), mutant xylanases obtained by random mutagenesis and mutant xylanases obtained by site-directed mutagenesis.

Variant polypeptides of the invention may also be subjected to further modifications that do not necessarily affect sensitivity to inhibitors or thermo-sensitivity, including any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant amino acid sequence retains xylanase activity, preferably having at least substantially the same xylanase activity as the unmodified sequence.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

Polypeptides of the invention also include fragments of the full-length sequences mentioned above having xylanase activity.

Polypeptides of the invention may further comprise heterologous amino acid sequences, typically at the N-terminus or C-terminus, preferably the N-terminus. Heterologous sequence may include sequences that affect intra or extracellular protein targeting (such as leader sequences).

Polypeptides of the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Polypeptides of the invention may also be produced as fusion proteins, for example to aid in extraction and purification. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences, such as a thrombin cleavage site. Preferably the fusion protein will not hinder the function of the protein sequence of interest.

The use of appropriate host cells is expected to provide for such post-translational modifications as may be needed to confer optimal biological activity on recombinant expression products of the invention.

Polypeptides of the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A polypeptide of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a polypeptide of the invention.

Variant polypeptides of the invention have an altered sensitivity to xylanase inhibitors and an altered thermo-sensitivity compared to the parent xylanase sequence – which may be a corresponding wild type xylanase. Preferably, variant polypeptides have reduced sensitivity to xylanase inhibitors and reduced thermo-sensitivity. The term "altered sensitivity to xylanase inhibitors" means that the extent to which the endo-β-1,4-xylanase activity of a variant polypeptide of the invention is inhibited by the xylanase inhibitor is different to that of the parent xylanase enzyme – which may be the corresponding wild type xylanase. Preferably the extent to which the variant polypeptide is inhibited by the inhibitor is less than that of the parent xylanase enzyme – which may be the wild type protein. This may, for example, be due to a change in the three-dimensional structure of the variant polypeptide such that the inhibitor no longer binds with the same affinity as it does to the parent xylanase enzyme – which may be the wild type enzyme. The term "altered thermo-sensitivity" means that the variant polypeptide of the invention has an altered thermostability or temperature optimum determined as described in the methods included below. This may, for example, be due to a change which increases the denaturing temperature of the polypeptide, i.e. that temperature at which the peptide is denatured, compared to the parent xylanase enzyme – which may be the wild type enzyme.

The sensitivity of the variant polypeptides of the invention to xylanase inhibitors can be assayed using the xylanase inhibitor assay described below. A suitable inhibitor for use in the assay is the inhibitor purified from wheat flour in Example 1. Other inhibitors are described below. The thermo-sensitivity of the variant polypeptide of the

invention may be described using the thermostability and temperature optimum assay described below.

Xylanase assay (Endo-β-1,4-Xylanase activity)

Xylanase samples are diluted in citric acid (0.1 M) - *di*-sodium-hydrogen phosphate (0.2 M) buffer, pH 5.0, to obtain approx. OD = 0.7 in the final assay. Three dilutions of the sample and an internal standard with a defined activity are thermostated for 5 minutes at 40°C. At time = 5 minutes, 1 Xylazyme tab (crosslinked, dyed xylan substrate) is added to the enzyme solution. At time = 15 minutes (or in some cases longer, depending on the xylanase activity present in the sample) the reaction is terminated, by adding 10 ml of 2% TRIS. The reaction mixture is centrifuged and the OD of the supernatant is measured at 590 nm. Taking into account the dilutions and the amount of xylanase, the activity (TXU, Total-Xylanase-Units) of the sample can be calculated relative to the standard.

Xylanase inhibitors

As used herein, the term "xylanase inhibitor" refers to a compound, typically a protein, whose role is to control the depolymerisation of complex carbohydrates, such as arabinoxylan, found in plant cell walls. These xylanase inhibitors are capable of reducing the activity of naturally occurring xylanase enzymes as well as those of fungal or bacterial origin. Although the presence of xylanase inhibitors have been reported in cereal seeds (see for example McLauchlan et al 1999a; Rouau and Suget 1998) their impact on the efficacy of xylanase enzymes has not been extensively examined.

McLauchlan et al (1999a) disclose the isolation and characterisation of a protein from wheat that binds to and inhibits two family-11 xylanases. Likewise, WO 98/49278 demonstrates the effect of a wheat flour extract on the activity of a group of microbial xylanases all of which are classified as family 11 xylanases. Debyser et al. (1999) also disclose that endoxylanases from *Aspergillus niger* and *Bacillus subtilis*, which are both members of the family 11 xylanases were inhibited by a wheat xylanase inhibitor

called TAXI (*Triticum aestivum* xylanase-inhibitor). McLauchlan *et al* (1999b) teach that extracts from commercial flours such as wheat, barley, rye and maize are capable of inhibiting both family 10 and 11 xylanases.

The xylanase inhibitor may be any suitable xylanase inhibitor. By way of example, the xylanase inhibitor may be the inhibitor described in WO-A-98/49278 and/or the xylanase inhibitor described by Rouau, X. and Surget, A. (1998), McLauchlan, R., et al. (1999b) and/or the xylanase inhibitor described in International Patent Publication No. WO00/39289.

In some applications, preferably the inhibitor is a TAXI inhibitor. As indicated above, the TAXI inhibitor is disclosed in, for example Debyser et al (1999) and/or WO00/39289. An example TAXI inhibitor may comprise one or more of the amino acid sequences presented as SEQ ID No's 13-19 in WO00/39289.

The inhibitor may be one or more inhibitors of the same type, for example TAXI. For some applications, the inhibitor may be a mixture of inhibitors of different types, for example TAXI and XIP (Xylanase inhibitor proteins).

Xylanase inhibitor assay

100 µl of an candidate inhibitor fraction, 250 µl xylanase solution (containing 12 TXU microbial xylanase/ml) and 650 µl buffer (0.1 M citric acid - 0.2M *di*-sodium hydrogen phosphate buffer, pH 5.0) are mixed. The mixture is thermostated for 5 minutes at 40.0°C. At time = 5 minutes one Xylazyme tab is added. At time = 15 minutes the reaction is terminated by adding 10 ml 2% TRIS. The reaction mixture is centrifuged (3500 g, 10 minutes, room temperature) and the supernatant is measured at 590 nm. The inhibition is calculated as residual activity compared to the blank. The blank is prepared the same way, except that the 100 µl inhibitor is substituted with 100 µl buffer (0.1 M citric acid - 0.2 M *di*-sodium hydrogen phosphate buffer, pH 5.0).

Specific xylanase inhibitor

As indicated, a xylanase inhibitor that may be used in accordance with the present invention is the xylanase inhibitor described in International Patent Publication No. WO00/39289.

This endogenous endo-β-1,4-xylanase inhibitor is obtainable from wheat flour. The inhibitor is a di-peptide, having a MW of about 40 kDa (as measured by SDS-PAGE or mass spectrometry) and a pI of about 8 to about 9.5.

Sequence analysis to date has revealed the that the inhibitor has at least one or more of the sequences presented as SEQ ID No. 2, SEQ ID No. 3, SEQ ID No 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7 and/or SEQ ID No. 8.

These inhibitors described in the prior art may also be used in assays to determine the sensitivity of a variant polypeptide of the invention to xylanase inhibitors. They may also be used as described below to modulate the functionality of a xylanase.

Thermostability assay

Solutions of the variant polypeptide are incubated for 10 to 240 minutes, preferably 30 to 240 minutes, at temperatures varying from 30 to 80°C, preferably at 50, 55, 60 or 65°C. Hereafter the xylanase solutions are assayed as described in "Xylanase assay" above. Activities as a function of different pre-incubation temperatures are expressed relatively to the highest activity obtained.

Temperature optimum assay.

Solutions of the variant polypeptides are assayed as described in "Xylanase assay", except for the temperature. Instead of only assaying the xylanase variants at 40°C, the xylanases may be incubated and assayed at one or more of the following temperatures:

30, 40, 50, 60 and 70°C. Hereby it is possible to determine a temperature optimum for the xylanase variants.

Polynucleotides

Polynucleotides of the invention comprise nucleic acid sequences encoding the variant polypeptide sequences of the invention. It will be understood by a skilled person that numerous different polynucleotides can encode the same polypeptide as a result of the degeneracy of the genetic code. In addition, it is to be understood that skilled persons may, using routine techniques, make nucleotide substitutions that do not affect the polypeptide sequence encoded by the polynucleotides of the invention to reflect the codon usage of any particular host organism in which the polypeptides of the invention are to be expressed.

Polynucleotides of the invention may comprise DNA or RNA. They may be single-stranded or double-stranded. They may also be polynucleotides which include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the polynucleotides described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of polynucleotides of the invention.

Nucleotide vectors and host cells

Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides of the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the

host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast and fungi.

Preferably, a polynucleotide of the invention in a vector is operably linked to a regulatory sequence which is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" refers to a juxtaposition wherein the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under conditions compatible with the control sequences. The term "regulatory sequences" includes promoters and enhancers and other expression regulation signals.

Enhanced expression of the polynucleotide encoding the polypeptide of the invention may also be achieved by the selection of heterologous regulatory regions, e.g. promoter, secretion leader and terminator regions, which serve to increase expression and, if desired, secretion levels of the protein of interest from the chosen expression host and/or to provide for the inducible control of the expression of the polypeptide of the invention.

Aside from the promoter native to the gene encoding the polypeptide of the invention, other promoters may be used to direct expression of the polypeptide of the invention. The promoter may be selected for its efficiency in directing the expression of the polypeptide of the invention in the desired expression host.

In another embodiment, a constitutive promoter may be selected to direct the expression of the desired polypeptide of the invention. Examples of strong constitutive and/or inducible promoters which are preferred for use in fungal expression hosts are those which are obtainable from the fungal genes for xylanase (*xlnA*), phytase, ATP-synthetase, subunit 9 (*oliC*), triose phosphate isomerase (*tpi*), alcohol

dehydrogenase (*Adh*A), α-amylase (*amy*), amyloglucosidase (AG - from the *gla*A gene), acetamidase (*amd*S) and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) promoters.

Examples of strong yeast promoters are those obtainable from the genes for alcohol dehydrogenase, lactase, 3-phosphoglycerate kinase and triosephosphate isomerase.

Examples of strong bacterial promoters are the α-amylase and *SP02* promoters as well as promoters from extracellular protease genes.

Hybrid promoters may also be used to improve inducible regulation of the expression construct.

Often, it is desirable for the polypeptide of the invention to be secreted from the expression host into the culture medium from where the polypeptide of the invention may be more easily recovered. According to the present invention, the polypeptide of the invention's native secretion leader sequence may be used to effect the secretion of the expressed polypeptide of the invention. However, an increase in the expression of the polypeptide of the invention sometimes results in the production of the protein in levels beyond that which the expression host is capable of processing and secreting, creating a bottleneck such that the protein product accumulates within the cell. Accordingly, the present invention also provides heterologous leader sequences to provide for the most efficient secretion of the polypeptide of the invention from the chosen expression host.

According to the present invention, the secretion leader may be selected on the basis of the desired expression host. A heterologous secretion leader may be chosen which is homologous to the other regulatory regions of the expression construct. For example, the leader of the highly secreted amyloglucosidase (AG) protein may be used in combination with the amyloglucosidase (AG) promoter itself, as well as in

combination with other promoters. Hybrid signal sequences may also be used with the context of the present invention.

Examples of preferred heterologous secretion leader sequences are those originating from the fungal amyloglucosidase (AG) gene (*gla*A - both 18 and 24 amino acid versions e.g. from *Aspergillus*), the α-factor gene (yeasts e.g. *Saccharomyces* and *Kluyveromyces*) or the α-amylase gene (*Bacillus*).

Such vectors may be transformed into a suitable host cell as described above to provide for expression of a polypeptide of the invention. Thus, in a further aspect the invention provides a process for preparing polypeptides according to the invention which comprises cultivating a host cell transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the polypeptides, and recovering the expressed polypeptides. Suitable host cells include, for example, fungal cells, such as *Aspergillus* and yeast cells, such as yeast cells of the genus *Kluyveromyces* or *Saccharomyces*. Other suitable host cells are discussed below.

The vectors may be for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes. The most suitable selection systems for industrial micro-organisms are those formed by the group of selection markers which do not require a mutation in the host organism. Examples of fungal selection markers are the genes for acetamidase (*amd*S), ATP synthetase, subunit 9 (*oli*C), orotidine-5'-phosphate-decarboxylase (*pyr*A), phleomycin and benomyl resistance (benA). Examples of non-fungal selection markers are the bacterial G418 resistance gene (this may also be used in yeast, but not in fungi), the ampicillin resistance gene (*E. coli*), the neomycin resistance gene (*Bacillus*) and the *E. coli uid*A gene, coding for β-glucuronidase (GUS). Vectors may be used *in vitro*, for example for the production of RNA or used to transfect or transform a host cell.

A further embodiment of the invention provides host cells transformed or transfected with a polynucleotide of the invention. Preferably said polynucleotide is carried in a vector for the replication and expression of said polynucleotides. The cells will be chosen to be compatible with the said vector and may for example be prokaryotic (for example bacterial), fungal, yeast or plant cells.

Bacteria from the genus *Bacillus* are very suitable as heterologous hosts because of their capability to secrete proteins into the culture medium. Other bacteria suitable as hosts are those from the genera *Streptomyces* and *Pseudomonas*.

Depending on the nature of the polynucleotide encoding the polypeptide of the invention, and/or the desirability for further processing of the expressed protein, eukaryotic hosts such as yeasts or fungi may be preferred. In general, yeast cells are preferred over fungal cells because they are easier to manipulate. However, some proteins are either poorly secreted from the yeast cell, or in some cases are not processed properly (e.g. hyperglycosylation in yeast). In these instances, a fungal host organism should be selected.

A heterologous host may also be chosen wherein the polypeptide of the invention is produced in a form which is substantially free from other xylanases. This may be achieved by choosing a host which does not normally produce such enzymes.

Examples of preferred expression hosts within the scope of the present invention are fungi such as *Aspergillus* species and *Trichoderma* species; bacteria such as *Bacillus* species, *Streptomyces* species and *Pseudomonas* species; and yeasts such as *Kluyveromyces* species and *Saccharomyces* species.

Particularly preferred expression hosts may be selected from *Aspergillus niger*, *Aspergillus niger var. tubigenis*, *Aspergillus niger var. awamori*, *Aspergillus aculeatis*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Trichoderma reesei*, *Bacillus*

subtilis, Bacillus licheniformis, Bacillus amyloliquefaciens, Kluyveromyces lactis, Lactococcus lactis, Thermomyces (Humicola) lanuginosus, Pichia spp., *Schizosaccheromyces pombe, Mucor* spp. and *Saccharomyces cerevisiae.*

According to the present invention, the production of the polypeptide of the invention can be effected by the culturing of microbial expression hosts, which have been transformed with one or more polynucleotides of the present invention, in a conventional nutrient fermentation medium.

The fermentation medium can comprise a known culture medium containing a carbon source (e.g. glucose, maltose, molasses, etc.), a nitrogen source (e.g. ammonium sulphate, ammonium nitrate, ammonium chloride, etc.), an organic nitrogen source (e.g. yeast extract, malt extract, peptone, etc.) and inorganic nutrient sources (e.g. phosphate, magnesium, potassium, zinc, iron, etc.). Optionally, an inducer may be added.

The selection of the appropriate medium may be based on the choice of expression hosts and/or based on the regulatory requirements of the expression construct. Such media are well known to those skilled in the art. The medium may, if desired, contain additional components favouring the transformed expression hosts over other potentially contaminating microorganisms.

After fermentation, the cells can be removed from the fermentation broth by means of centrifugation or filtration. After removal of the cells, the variant polypeptide of the invention may then be recovered and, if desired, purified and isolated by conventional means.

Organisms

The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the variant xylanase protein according

to the present invention and/or products obtained therefrom, wherein a transcriptional regulatory sequence can allow expression of the nucleotide sequence according to the present invention when present in the organism. Suitable organisms may include a prokaryote, fungus, yeast or a plant. For the xylanase aspect of the present invention, a preferable organism may be a bacterium, preferably of the genus Bacillus, more preferably *Bacillus subtilis*.

The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the protein according to the present invention and/or products obtained therefrom, wherein the transcriptional regulatory sequence can allow expression of the nucleotide sequence according to the present invention within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

The term "transgenic organism" does not cover native nucleotide coding sequences in their natural environment when they are under the control of their native promoter which is also in its natural environment.

Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the amino acid sequence according to the present invention, constructs according to the present invention (including combinations thereof), vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention or the products thereof. The transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from, the cell or organism.

Transformation of Host Cells/Host Organisms

As indicated earlier, the host organism can be a prokaryotic or a eukaryotic organism. Examples of suitable prokaryotic hosts include *E. coli* and *Bacillus subtilis*. Teachings

on the transformation of prokaryotic hosts is well documented in the art, for example see Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd edition, 1989, Cold Spring Harbor Laboratory Press) and Ausubel *et al.*, Short Protocols in Molecular Biology (1999), 4th Ed., John Wiley & Sons, Inc.

If a prokaryotic host is used then the nucleotide sequence may need to be suitably modified before transformation - such as by removal of introns.

As mentioned above, a preferred host organism is of the genus Bacillus, such as *Bacillus subtilis*.

In another embodiment the transgenic organism can be a yeast. In this regard, yeasts have also been widely used as a vehicle for heterologous gene expression. The species *Saccharomyces cerevisiae* has a long history of industrial use, including its use for heterologous gene expression. Expression of heterologous genes in *Saccharomyces cerevisiae* has been reviewed by Goodey et al (1987, Yeast Biotechnology, D R Berry et al, eds, pp 401-429, Allen and Unwin, London) and by King et al (1989, Molecular and Cell Biology of Yeasts, E F Walton and G T Yarronton, eds, pp 107-133, Blackie, Glasgow).

For several reasons *Saccharomyces cerevisiae* is well suited for heterologous gene expression. First, it is non-pathogenic to humans and it is incapable of producing certain endotoxins. Second, it has a long history of safe use following centuries of commercial exploitation for various purposes. This has led to wide public acceptability. Third, the extensive commercial use and research devoted to the organism has resulted in a wealth of knowledge about the genetics and physiology as well as large-scale fermentation characteristics of *Saccharomyces cerevisiae*.

A review of the principles of heterologous gene expression in Saccharomyces cerevisiae and secretion of gene products is given by E Hinchcliffe E Kenny (1993, "Yeast as a

vehicle for the expression of heterologous genes", Yeasts, Vol 5, Anthony H Rose and J Stuart Harrison, eds, 2nd edition, Academic Press Ltd.).

Several types of yeast vectors are available, including integrative vectors, which require recombination with the host genome for their maintenance, and autonomously replicating plasmid vectors.

In order to prepare the transgenic *Saccharomyces*, expression constructs are prepared by inserting the nucleotide sequence of the present invention into a construct designed for expression in yeast. Several types of constructs used for heterologous expression have been developed. The constructs contain a promoter active in yeast fused to the nucleotide sequence of the present invention, usually a promoter of yeast origin, such as the GAL1 promoter, is used. Usually a signal sequence of yeast origin, such as the sequence encoding the SUC2 signal peptide, is used. A terminator active in yeast ends the expression system.

For the transformation of yeast several transformation protocols have been developed. For example, a transgenic *Saccharomyces* according to the present invention can be prepared by following the teachings of Hinnen et al (1978, Proceedings of the National Academy of Sciences of the USA 75, 1929); Beggs, J D (1978, Nature, London, 275, 104); and Ito, H et al (1983, J Bacteriology 153, 163-168).

The transformed yeast cells are selected using various selective markers. Among the markers used for transformation are a number of auxotrophic markers such as LEU2, HIS4 and TRP1, and dominant antibiotic resistance markers such as aminoglycoside antibiotic markers, e.g. G418.

Another host organism is a plant. The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

A transgenic plant of the invention may be produced from any plant such as the seed-bearing plants (angiosperms), and conifers. Angiosperms include dicotyledons and monocotyledons. Examples of dicotyledonous plants include tobacco, (*Nicotiana plumbaginifolia* and *Nicotiana tabacum*), arabidopsis (*Arabidopsis thaliana*), *Brassica napus*, *Brassica nigra*, *Datura innoxia*, *Vicia narbonensis*, *Vicia faba*, pea (*Pisum sativum*), cauliflower, carnation and lentil (*Lens culinaris*). Examples of monocotyledonous plants include cereals such as wheat, barley, oats and maize.

Techniques for producing transgenic plants are well known in the art. Typically, either whole plants, cells or protoplasts may be transformed with a suitable nucleic acid construct encoding a zinc finger molecule or target DNA (see above for examples of nucleic acid constructs). There are many methods for introducing transforming DNA constructs into cells, but not all are suitable for delivering DNA to plant cells. Suitable methods include *Agrobacterium* infection (see, among others, Turpen *et al.*, 1993, J. Virol. Methods, 42: 227-239) or direct delivery of DNA such as, for example, by PEG-mediated transformation, by electroporation or by acceleration of DNA coated particles. Acceleration methods are generally preferred and include, for example, microprojectile bombardment. A typical protocol for producing transgenic plants (in particular monocotyledons), taken from U.S. Patent No. 5, 874, 265, is described below.

An example of a method for delivering transforming DNA segments to plant cells is microprojectile bombardment. In this method, non-biological particles may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly stably transforming both dicotyledons and monocotyledons, is that neither the isolation of protoplasts nor the susceptibility to *Agrobacterium* infection is required. An illustrative embodiment of a method for

delivering DNA into plant cells by acceleration is a Biolistics Particle Delivery System, which can be used to propel particles coated with DNA through a screen, such as a stainless steel or Nytex screen, onto a filter surface covered with plant cells cultured in suspension. The screen disperses the tungsten-DNA particles so that they are not delivered to the recipient cells in large aggregates. It is believed that without a screen intervening between the projectile apparatus and the cells to be bombarded, the projectiles aggregate and may be too large for attaining a high frequency of transformation. This may be due to damage inflicted on the recipient cells by projectiles that are too large.

For the bombardment, cells in suspension are preferably concentrated on filters. Filters containing the cells to be bombarded are positioned at an appropriate distance below the macroprojectile stopping plate. If desired, one or more screens are also positioned between the gun and the cells to be bombarded. Through the use of techniques set forth herein one may obtain up to 1000 or more clusters of cells transiently expressing a marker gene ("foci") on the bombarded filter. The number of cells in a focus which express the exogenous gene product 48 hours post-bombardment often range from 1 to 10 and average 2 to 3.

After effecting delivery of exogenous DNA to recipient cells by any of the methods discussed above, a preferred step is to identify the transformed cells for further culturing and plant regeneration. This step may include assaying cultures directly for a screenable trait or by exposing the bombarded cultures to a selective agent or agents.

An example of a screenable marker trait is the red pigment produced under the control of the R-locus in maize. This pigment may be detected by culturing cells on a solid support containing nutrient media capable of supporting growth at this stage, incubating the cells at, e.g., 18°C and greater than 180 $\mu E\ m^{-2}\ s^{-1}$, and selecting cells from colonies (visible aggregates of cells) that are pigmented. These cells may be cultured further, either in suspension or on solid media.

An exemplary embodiment of methods for identifying transformed cells involves exposing the bombarded cultures to a selective agent, such as a metabolic inhibitor, an antibiotic, herbicide or the like. Cells which have been transformed and have stably integrated a marker gene conferring resistance to the selective agent used, will grow and divide in culture. Sensitive cells will not be amenable to further culturing.

To use the bar-bialaphos selective system, bombarded cells on filters are resuspended in nonselective liquid medium, cultured (e.g. for one to two weeks) and transferred to filters overlaying solid medium containing from 1-3 mg/l bialaphos. While ranges of 1-3 mg/l will typically be preferred, it is proposed that ranges of 0.1-50 mg/l will find utility in the practice of the invention. The type of filter for use in bombardment is not believed to be particularly crucial, and can comprise any solid, porous, inert support.

Cells that survive the exposure to the selective agent may be cultured in media that supports regeneration of plants. Tissue is maintained on a basic media with hormones for about 2-4 weeks, then transferred to media with no hormones. After 2-4 weeks, shoot development will signal the time to transfer to another media.

Regeneration typically requires a progression of media whose composition has been modified to provide the appropriate nutrients and hormonal signals during sequential developmental stages from the transformed callus to the more mature plant. Developing plantlets are transferred to soil, and hardened, e.g., in an environmentally controlled chamber at about 85% relative humidity, 600 ppm CO_2, and 250 $\mu E\ m^{-2}\ s^{-1}$ of light. Plants are preferably matured either in a growth chamber or greenhouse. Regeneration will typically take about 3-12 weeks. During regeneration, cells are grown on solid media in tissue culture vessels. An illustrative embodiment of such a vessel is a petri dish. Regenerating plants are preferably grown at about 19°C to 28°C. After the regenerating plants have reached the stage of shoot and root development, they may be transferred to a greenhouse for further growth and testing.

Genomic DNA may be isolated from callus cell lines and plants to determine the presence of the exogenous gene through the use of techniques well known to those skilled in the art such as PCR and/or Southern blotting.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a construct encoding a variant xylanase polypeptide according to the present invention and which is capable of introducing the construct into the genome of a plant.

The vector system may comprise one vector, but it can comprise at least two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* (An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. *et al.* (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208).

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

B. Uses

In a general sense, a variant xylanase of the invention may be used to alter, for example reduce, the viscosity derived from the presence of hemicellulose or arabinoxylan in a solution or system comprising plant cell wall material. Typically said plant cell wall materials will comprise one or more xylanase inhibitors. Suitably said plant cell wall materials will be processed at a preferred temperature.

Specifically, a variant xylanase of the invention may be used in processing plant materials for use as foodstuffs, such as animal feed, in processing cereals, in starch production, in baking and in the processing of wood pulp to make paper.

The variant xylanase of the present invention may be used in the preparation of a dough and baked products that are obtained by baking such a dough, and in the preparation of noodle and pasta products.

The variant xylanase of the present invention may be used in a dough improving composition comprising.

Preparation of foodstuffs

A variant xylanase of the invention may be used to process plant materials such as cereals that are used in foodstuffs including animal feed. As used herein, the term "cereal" means any kind of grain used for food and/or any grass producing this grain such as but not limited to any one of wheat, milled wheat, barley, maize, sorghum, rye, oats, triticale and rice or combinations thereof. In one preferred embodiment, the cereal is a wheat cereal.

The xylan in the food and/or feed supplement is modified by contacting the xylan with the variant xylanase of the present invention.

As used herein, the term "contacting" includes but is not limited to spraying, coating, impregnating or layering the food and/or feed supplement with the variant xylanase enzyme of the present invention.

In one embodiment, the food and/or feed supplement of the present invention may be prepared by mixing the variant xylanase enzyme directly with a food and/or feed supplement. By way of example, the variant xylanase enzyme may be contacted (for example, by spraying) onto a cereal-based food and/or feed supplement such as milled wheat, maize or soya flour.

It is also possible to incorporate the variant xylanase enzyme into a second (and different) food and/or feed or drinking water which is then added to the food and/or feed supplement of the present invention. Accordingly, it is not essential that the variant xylanase enzyme provided by the present invention is incorporated into the cereal-based food and/or feed supplement itself, although such incorporation forms a particularly preferred aspect of the present invention.

In one embodiment of the present invention, the food and/or feed supplement may be combined with other food and/or feed components to produce a cereal-based food and/or feed. Such other food and/or feed components may include one or more other (preferably thermostable) enzyme supplements, vitamin food and/or feed supplements, mineral food and/or feed supplements and amino acid food and/or feed supplements. The resulting (combined) food and/or feed supplement comprising possibly several different types of compounds can then be mixed in an appropriate amount with the other food and/or feed components such as cereal and protein supplements to form a human food and/or an animal feed.

In one preferred embodiment, the food and/or feed supplement of the present invention can be prepared by mixing different enzymes having the appropriate activities to produce an enzyme mix. By way of example, a cereal-based food and/or feed supplement formed from e.g. milled wheat or maize may be contacted (e.g. by

spraying) either simultaneously or sequentially with the xylanase enzyme and other enzymes having appropriate activities. These enzymes may include but are not limited to any one or more of an amylase, a glucoamylase, a mannanase, an a galactosidase, a phytase, a lipase, a galactolipase, a phospholipase, a glucanase, an arabinofuranosidase, a pectinase, a protease, a glucose oxidase, a hexose oxidase and a xylanase. Enzymes having the desired activities may for instance be mixed with the xylanase of the present invention either before contacting these enzymes with a cereal-based food and/or feed supplement or alternatively such enzymes may be contacted simultaneously or sequentially on such a cereal based supplement. The food and/or feed supplement is then in turn mixed with a cereal-based food and/or feed to prepare the final food and/or feed. It is also possible to formulate the food and/or feed supplement as a solution of the individual enzyme activities and then mix this solution with a food and/or feed material prior to processing the food and/or feed supplement into pellets or as a mash.

The variant xylanase of the present invention may be used in combination with other components. Thus, the present invention also relates to combinations.

The combination of the present invention comprises the variant of the present invention and another component which is suitable for animal or human consumption.

Further examples of other suitable components include one or more of: thickeners, gelling agents, emulsifiers, binders, crystal modifiers, sweeteners (including artificial sweeteners), rheology modifiers, stabilisers, anti-oxidants, dyes, enzymes, carriers, vehicles, excipients, diluents, lubricating agents, flavouring agents, colouring matter, suspending agents, disintegrants, granulation binders etc. These other components may be natural. These other components may be prepared by use of chemical and/or enzymatic techniques.

As used herein the term "component suitable for animal or human consumption" means a compound which is or can be added to the composition of the present

invention as a supplement which may be of nutritional benefit, a fibre substitute or have a generally beneficial effect to the consumer.

Bakery products

The present invention provides the use of a variant xylanase polypeptide of the invention in a process for preparing a foodstuff. Typical bakery (baked) products in accordance with the present invention include bread - such as loaves, rolls, buns, pizza bases etc. - pretzels, tortillas, cakes, cookies, biscuits, crackers etc. The preparation of foodstuffs such as bakery products is well known in the art.

The present invention advantageously provides a variant xylanase polypeptide capable of improving the properties of flour based doughs and products made from such doughs. This is, in respect of baked products, achieved by providing a method for preparing baked products which have highly desirable characteristics with respect to bread volume, crumb structure and appearance and which additionally have an extended shelf life as reflected in an enhanced softness, i.e. the staling of the baked products is retarded relative to a baked product made without use of the enzyme of the invention. Although it is presently preferred to use the method for the manufacturing of yeast leavened bread products such as bread loaves, rolls or toast bread, the use of the method for any other types of doughs and dough based products such as noodle and pasta products and cakes, the quality of which can be improved by the addition of the enzymes of the invention, is also contemplated.

The present invention further relates to pasta doughs, noodle doughs and cake doughs or batters comprising the variant xylanase polypeptide and finished products made from such doughs or batters.

Starch production

A variant xylanase of the invention may also be used in starch production from plant materials derived from cereals and tubers, such as potatoes.

Processing of wood pulp

A variant xylanase of the invention may also be used in processing wood pulp, for example in the preparation of paper.

The present invention will now be further described with reference to the following examples which are intended to be illustrative only and non-limiting.

EXAMPLES

Example 1 - Purification and characterisation of wheat endogenous xylanase inhibitor.

2 kg wheat flour (Danish reform, batch 99056) was extracted with water, using a flour:water ratio of 1:2, during 10 minutes of stirring. The soluble endogenous xylanase inhibitor was separated from the flour-water slurry by centrifugation. The extraction and centrifugation was performed at 4°C. The inhibitor was purified from the water extract by the following chromatographic techniques and concentration techniques: HPLC-SEC, HPLC-CIEC, rotary evaporation, HPLC-HIC, HPLC-SEC and rotary evaporation. The xylanase inhibitor could be monitored and quantified during purification, using the following quantification method.

Inhibitor quantification method

1 XIU (Xylanase Inhibitor Unit) is defined as the amount of inhibitor that decreases 1 TXU to 0.5 TXU under the conditions described below.

The xylanase used in this assay is *Bacillus subtilis* wild type xylanase.

250 µl xylanase solution containing 12 TXU/ml, approx. 100 µl xylanase inhibitor solution and citric acid (0.1 M) - *di*-sodium-hydrogen phosphate (0.2 M) buffer, pH 5, to react a reaction volume of 1000 µl is pre-incubated for 5 minutes at 40°C. At t = 5 minutes, 1 Xylazyme (Megazyme, Ireland) tablet is added to the reaction mixture. At t = 15 minutes the reaction is terminated, by addition of 10 ml 2 % TRIS/NaOH, pH 12. The solution is filtered and the absorbency of the supernatant is measured at 590 nm. By choosing several different concentrations of inhibitor in the above assay, it is possible to create a plot of OD versus inhibitor concentration. Using the slope (a) and intercept (b) from this plot and the concentration of the xylanase it is possible to calculate the amount of XIU in a given inhibitor solution (equation 1).

Equation 1 amount of XIU in solution = ((b/2)/-a)/ TXU

From the endogenous xylanase inhibitor purification, the following inhibitor yield was recovered (table 1).

Table 1. Wheat endogenous xylanase inhibitor recovery after purification.

Sample	Amount	XIU	XIU, total	Recovery, %
Flour	2000 g	590/g	1.180.000	100
Purified inhibitor	90 ml	4658/ml	419.220	35.5

The inhibitor sample was pure and free from wheat endogenous xylanolytic activities.

Example 2 - Site-directed mutagenesis on xylanases.

Specific mutants of *Bacillus subtilis* xylanase may be obtained by site directed mutagenesis of the wild type enzyme, by the use of any of a number of commercially available mutagenesis kits. An example of how to obtain the D11F mutant using the Quick Exchange kit, available from Stratagene Cloning Systems, 11011 North Torrey Pines Road, La Jolla, CA 92037, USA is given below :

The DNA sequence encoding *Bacillus subtilis* xylanase A has been published by Paice et al., 1986.

The sequence of the coding region is as follows, with the sequence encoding the mature part of the protein shown in capitals:

catatgtttaagtttaaaaagaatttcttagttggattatcggcagctttaatgagtatt
agcttgttttcggcaaccgcctctgcaGCTAGCACAGACTACTGGCAAAATTGGACTGAT
GGGGGCGGTATAGTAAACGCTGTCAATGGGTCTGGCGGGAATTACAGTGTTAATTGGTCT
AATACCGGAAATTTTGTTGTTGGTAAAGGTTGGACTACAGGTTCGCCATTTAGGACGATA
AACTATAATGCCGGAGTTTGGGCGCCGAATGGCAATGGATATTTAACTTTATATGGTTGG
ACGAGATCACCTCTCATAGAATATTATGTAGTGGATTCATGGGGTACTTATAGACCTACT
GGAACGTATAAAGGTACTGTAAAAAGTGATGGGGGTACATATGACATATATACAACTACA
CGTTATAACGCACCTTCCATTGATGGCGATCGCACTACTTTTACGCAGTACTGGAGTGTT
CGCCAGTCGAAGAGACCAACCGGAAGCAACGCTACAATCACTTTCAGCAATCATGTGAAC
GCATGGAAGAGCCATGGAATGAATCTGGGCAGTAATTGGGCTTACCAAGTCATGGCGACA
GAAGGATATCAAAGTAGTGGAAGTTCTAACGTAACAGTGTGGTAA

The part of the gene encoding the mature part of the wild type enzyme may be expressed intracellularly in *E.coli* by methods well known to people skilled in the art of molecular biology. For example :

1. Generating a copy of the capitalised part of the above described gene by use of the Polymerase Chain Reaction (PCR) with an added Nde1 restriction enzyme site

(CATATG) before the GCTAGCACA and an added HindIII restriction site (AAGCTT) after the GTGTGGTAA.

2. Inserting the resultant modified copy of the gene by use of the above mentioned enzymes into the expression vector pET24a(+), which can be obtained from Novagen, Inc. 601 Science Drive, Madison, WI 53711, USA.
3. Transforming into a suitable *E.coli* strain and expression by fermentation as described by the vendor of pET24a(+).

Our D11F mutant enzyme may be obtained by using the "Quick Exchange" mutagenesis kit according to the manufacturer, and using the above described *Bacillus subtilis* wild type xylanase-pET24a(+) construct and the following PCR mutagenesis primers :

Sense primer:
CTACTGGCAAAATTGGACTTTTGGAGGAGGTATAGTAAACGCTG

Antisense primer :
CAGCGTTTACTATACCTCCTCCAAAAGTCCAATTTTGCCAGTAG

The mutant enzyme is expressed and purified using the same protocols as for the wild type enzyme.

Example 3 - Inhibition studies of xylanase mutants.

Xylanase mutants expressed in *E. coli* (see Example 2) were fermented and purified (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

These pure xylanase mutant preparations were diluted to 12 TXU/ml using 0.1 M citric acid - 0.2 M *di*-sodium-hydrogen phosphate, pH 5.0 and used in the following assay.

A stable inhibitor preparation was made according to the protocol described in Example 1. This stable inhibitor preparation is used as stock for all xylanase-xylanase inhibitor studies. Using the inhibitor quantification method described in Example 1, the inhibitor preparation was analysed to contain 126 XIU/ml.

Assay

To 250 µl diluted xylanase mutant preparations, are added 0, 10, 25, 50 or 100 µl inhibitor preparation, respectively. To these inhibitor-xylanase mixtures were added 0.1 M citric acid - 0.2 M *di*-sodium-hydrogen phosphate, pH 5.0 making the end-volume 1000 µl. These reaction mixtures were pre-incubated for 5 minutes at 40°C. Hereafter 1 Xylazyme tablet (Megazyme, Ireland) were added to all inhibitor-xylanase mixtures. After 10 minutes of incubation at 40°C, the reactions were terminated, by adding 10 ml 2% Tris/NaOH, pH 12.0. The mixtures were centrifuged and the liberated blue colour from the substrate was measured at 590 nm.

The results in respect of one variant xylanase are presented in table 2.

Table 2. Relative inhibition of a xylanase mutant and parent xylanase (here wildtype enzyme) as a function of xylanase inhibitor.

	Xylanase Inhibitor (XIU/ml)				
Xylanase	0	1,26	3,15	6,3	12,6
	Relative inhibition, %				
Wildtype	100	77	48	29	23
Mutant ID : D121F/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N	100	101	88	70	50

From the results in Table 2, it can be seen that the variant xylanase has a changed sensitivity towards the wheat endogenous xylanase inhibitor.

Other suitable variant xylanases having changed inhibitor sensitivity include those based on *B. subtilis* xylanase with the following mutations:
i)D11F/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
ii)N32K/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
iii)T123D/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N

Example 4 - Thermostability studies of xylanase mutants.

Xylanase mutants expressed in *E. coli* (see Example 2) were fermented and purified (meaning no other xylanolytic activity were present in the purified preparation).

The xylanase mutants were analysed according to the "thermostability assay" mentioned above.

Suitable variant xylanases having altered thermostability include those based on *B. subtilis* xylanase with the following mutations:
i)D11F/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
ii)N32K/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R
iii)T123D/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N
iv)D121F/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N

Variant xylanases had an altered thermostability compared to the wild type xylanase.

Example 5. Temperature optimum studies of xylanase mutants.

Xylanase mutants expressed in *E. coli* (see Example 2) were fermented and purified (meaning no other xylanolytic activity were present in the purified preparation).

The xylanase mutants were analysed according to the "temperature optimum assay" mentioned above.

Suitable variant xylanases having altered thermostability include those based on *B. subtilis* xylanase with the following mutations:

i)D11F/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R

ii)N32K/K99N/T111M/T126Q/Y128F/A142V/T143S/T147S/K154R/H156K/N163S/M169L/S179R

iii)T123D/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N

iv)D121F/N8Y/S22P/N29T/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/P90A/T93N

Variant xylanases have changed temperature optima compared to the wild type xylanase.

Example 6. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (A)

A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo-sensitivity may be engineered by combining mutation D11F (changing inhibitor sensitivity) with mutations K99N, T111M, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R (changing thermo-sensitivity).

Example 7. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (B)

A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo-sensitivity may be engineered by combining mutation N32K (changing inhibitor sensitivity) with mutations K99N, T111M, T126Q, Y128F, A142V, T143S, T147S, K154R, H156K, N163S, M169L, S179R (changing thermo-sensitivity).

Example 8. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (C).

A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo sensitivity may be engineered by combining mutation T123D (changing inhibitor sensitivity) with mutations N8Y, S22P, N29T, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N (changing thermo-sensitivity).

Example 9. Xylanase variant having changed inhibitor-sensitivity and changed thermo-sensitivity – (D).

A variant of *Bacillus subtilis* 168 xylanase A, with changed inhibitor sensitivity and with altered thermo sensitivity may be engineered by combining mutation D121F (changing inhibitor sensitivity) with mutations N8Y, S22P, N29T, T44V, F48N, T50V, V57I, A59E, N61S, S74N, P75A, P90A, T93N (changing thermo-sensitivity).

Example 10. Inhibition studies of xylanase variants

B. subtilis xylanase mutants were obtained by site directed mutagenesis of the wild type enzyme (see example 2). The following variants were used in this example:

Mut 1:

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/D121F

Mut 2:

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/T123D

These mutants were expressed in *E. coli* (see Example 2), fermented and purified (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

These pure xylanase mutant preparations were evaluated for inhibitor sensitivity, using the "Xylanase inhibitor assay" mentioned previously.

A stable inhibitor preparation was made according to the protocol described in Example 1. This stable inhibitor preparation was used as stock for all xylanase-xylanase inhibitor studies. Using the "inhibitor quantification method" described in example 1, the inhibitor preparation was analysed to contain 126 XIU/ml.

The results are presented in Table 3.

Table 3. Relative inhibition of xylanase mutants and a parent xylanase (here wild type *B. subtilis* enzyme) as a function of xylanase inhibitor concentration, XIU/ml. The activity is expressed as a percentage of the activity at a given inhibitor concentration relative to the activity when XIU/ml =0.

	Inhibitor concentration, XIU/ml				
Xylanase	**0**	**1,26**	**3,15**	**6,3**	**12,6**
Wt	100	76	50	30	23
Mut 1	100	92	73	51	33

As can be seen from Table 3, the mutations introduced in the parent xylanase changed the sensitivity of the variant xylanases towards the wheat endogenous xylanase inhibitor.

In addition, the xylanase mutant (Mut 2) has was less stable to the wheat endogenous xylanase inhibitor at the concentration 1,26 XIU/ml.

Example 11. Thermostability studies of xylanase mutants

B. subtilis xylanase mutants were obtained by site directed mutagenesis of the wild type enzyme (see example 2). The following variants were used in this example:

Mut 1:
S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/D121F

Mut 2:
S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/T123D

These mutants were expressed in *E. coli* (see Example 2), fermented and purified (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

These xylanase mutant preparations were evaluated for thermostability, using the "thermostability assay" mentioned previously.

The results are presented in Tables 4 -7.

Table 4. Thermostability of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 50°C. The results are represented

as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	89	106	99
1	91	101	102
2	88	105	100
3	86	103	100
4	87	104	97

Table 5. Thermostability of parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 55°C. The results are represented as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	60	105	99
1	40	102	92
2	30	101	95
3	23	102	88
4	18	99	86

Table 6. Thermostability of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 60°C. The results are represented as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	-	92	101
1	-	75	85
2	-	56	68
3	-	44	50
4	-	33	39

Table 7. Thermostability of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof as a function of incubation time at 65°C. The results are represented as a percentage of the activity at given incubation time relative to the activity when time = 0 hour.

Time (hr)	wt	Mut 1	Mut 2
0	100	100	100
0.5	-	2	4
1	-	-	-
2	-	-	-
3	-	-	-
4	-	-	-

As can be seen from Tables 4–7, the mutations introduced in the parent xylanase changed the sensitivity of the variant xylanases to temperature (thermostability). At approx. 60°C two of the variants are more stable than the parent xylanase (here wild type).

Example 12. Temperature optimum studies of xylanase mutants.

B. subtilis xylanase mutants were obtained by site directed mutagenesis of the wild type enzyme (see example 2). The following variants were used in this example:

Mut 1:

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/D121F

Mut 2:

S2A/N8Y/I15M/S22P/N29T/S31Q/T44V/F48N/T50V/V57I/A59E/N61S/S74N/P75A/T123D

These mutants were expressed in *E. coli* (see Example 2), fermented and purified (meaning no other xylanolytic activity was present in the purified preparation) using a de-salting step and a cation exchange chromatography step.

These pure xylanase mutant preparations were evaluated for temperature optimum, using the "temperature optimum assay" mentioned previously.

The results are presented in Table 8.

Table 8. Temperature optimum of a parent xylanase (here wild type *B. subtilis* xylanase) and variants thereof. The results are represented as a percentage of the activity at a given temperature relative to the highest activity.

Temp (°C)	wt	Mut 1	Mut 2
30	37	22	27
40	66	46	51
50	100	86	87
60	17	100	100

As can be seen from Table 8, the mutations introduced in the parent xylanase changed the temperature optimum profile of the variant xylanases compared to the parent xylanase (here wild type *B. subtilis*). The variants have an approx. 10°C higher optimum than the parent xylanase.

SUMMARY

In summary, the present invention provides a means for altering the inhibitor sensitivity of a xylanase enzyme combined with a means for changing the thermostability and/or the temperature optimum of the enzyme. In this way, the inhibitor sensitivity and the thermosensitivity of the xylanase enzyme can be independently controlled.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

References

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Rouau, X. and Surget, A. (1998). Evidence for the presence of a pentosanase inhibitor in wheat flour. Journal of Cereal Science. **28**, 63-70.

Sibbesen,O. and Soerensen, J.F. (1999). Bacterial xylanase. UK A 9828599.2.

SEQUENCE LISTINGS

The amino acid sequence of the mature *Bacillus subtilis* xylanase (SEQ ID. No. 1).

```
1       10 11       20 21       30 31       40 41       50 51       60
ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT INYNAGVWAP

61      70 71       80 81       90 91      100 101     110 111     120
NGNGYLTLYG WTRSPLIEYY VVDSWGTYRP TGTYKGTVKS DGGTYDIYTT TRYNAPSIDG

121     130 131     140 141     150 151    160  161     170 171     180
DRTTFTQYWS VRQSKRPTGS NATITFSNHV NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS

181
NVTVW
```

Amino acid sequences derived from wheat flour xylanase inhibitor

A chain of inhibitor

N-terminal:

GAPVARAVEAVAPFGVCYDTKTLGNNLGGYAVPNV (35aa) SEQ ID NO. 2

C-terminal:

KRLGFSRLPHFTGCGGL (17aa) SEQ ID NO. 3

B chain of inhibitor

N-terminal:

LPVPAPVTKDPATSLYTIPFH (21aa) SEQ ID NO. 4

Lys-C digested B Chain:

LLASLPRGSTGVAGLANSGLALPAQVASAQK (31aa) SEQ ID NO. 5

GGSPAHYISARFIEVGDTRVPSVE (24aa) SEQ ID NO. 6

VNVGVLAACAPSK (13aa) SEQ ID NO. 7

VANRFLLCLPTGGPGVAIFGGGPVPWPQFTQSMPYTLVVVK SEQ ID NO. 8

CLAIMS

1. A method of altering the sensitivity of a xylanase polypeptide to an inhibitor and of altering the thermo-sensitivity of said xylanase polypeptide, which method comprises modifying a parent xylanase polypeptide to provide a variant xylanase polypeptide, and testing the sensitivity of said variant xylanase polypeptide to a xylanase inhibitor compared with the parent xylanase enzyme and testing the thermosensitivity of said variant xylanase polypeptide compared with the parent xylanase enzyme and selecting a variant xylanase polypeptide having an altered sensitivity to a xylanase inhibitor and an altered thermo-sensitivity compared with the parent xylanase enzyme.

2. A method according to claim 1 wherein said variant polypeptide is derived from a family 11 xylanase.

3. A method according to claim 1 wherein there are at least two of said amino acid modifications.

4. A method according to any one of the preceding claims wherein said amino acid modification is at any one or more of amino acid residues:

2, 8, 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 113, 114, 119, 120, 121, 122, 123, 124, 126, 128, 142, 143, 147, 154, 156, 163, 169, 175 and 179, or combinations hereof, based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

5. A method according to any one of the preceding claims wherein the inhibitor is an inhibitor found naturally in plant tissues.

6. A method according to any one of the preceding claims wherein the sensitivity to an inhibitor is reduced.

7. A variant xylanase polypeptide obtainable by the method according to any one of claims 1 to 6.

8. A variant xylanase polypeptide, or fragment thereof having xylanase activity, comprising one or more amino acid modifications such that the polypeptide or fragment thereof has an altered sensitivity to a xylanase inhibitor as compared with a parent xylanase enzyme and has an altered thermo-sensitivity as compared with a parent xylanase enzyme.

9. A variant polypeptide according to claim 8 which is derived from a family 11 xylanase.

10. A variant xylanase polypeptide, or fragment thereof having xylanase activity, according to any one of claims 8-9 wherein there are at least two of said amino acid modifications.

11. A variant xylanase polypeptide, or fragment thereof having xylanase activity according to any one of claims 8-10 wherein said amino acid modification is at any one or more of amino acid residues:

2, 8, 11, 12, 13, 15, 17, 22, 29, 31, 32, 34, 44, 48, 50, 57, 59, 61, 74, 75, 90, 93, 99, 111, 113, 114, 119, 120, 121, 122, 123, 124, 126, 128, 142, 143, 147, 154, 156, 163, 169, 175 and 179, or combinations thereof, based on the amino acid numbering of *B. subtilis* xylanase shown as SEQ ID No. 1, or the equivalent residues in other homologous xylanase polypeptides.

12. A variant xylanase polypeptide according to any one of claims 8-11 wherein the inhibitor is an inhibitor found naturally in plant tissues.

13. A variant xylanase polypeptide according to any one of claims 8-12 wherein the sensitivity to an inhibitor is reduced.

14. A method of altering the sensitivity of a xylanase polypeptide to an inhibitor and of altering the thermo-sensitivity of said xylanase polypeptide enzyme, which method comprises modifying one or more amino acid residues of said enzyme such that the polypeptide or a fragment thereof has an altered sensitivity to a xylanase inhibitor and has an altered thermo-sensitivity as compared with the parent xylanase enzyme.

15. A method according to claim 14 wherein said variant polypeptide is that defined in any one of claims 8 to 13.

16. A method according to claim 14 or claim 15 wherein the sensitivity of the enzyme to an inhibitor is reduced.

17. A composition comprising a variant polypeptide according to any one of claims 7 to 13.

18. A method of degrading or modifying a plant cell wall which method comprises contacting said plant cell wall with a polypeptide according to any one of claims 7 to 13 or a composition according to claim 17.

19. A method of processing a plant material which method comprises contacting said plant material with a polypeptide according to any one of claims 7 to 13 or composition according to claim 17.

20. A nucleotide sequence encoding a variant polypeptide according to any one of claims 7 to 13.

21. A construct comprising the nucleotide sequence according to claim 20.

22. Use of a variant polypeptide according to any one of claims 7 to 13 in a method of modifying plant materials.

23. Use of a variant polypeptide according to any one of claims 7 to 13 in any one or more of: baking, processing cereals, starch production, in processing wood, enhancing the bleaching of wood pulp.

Figure 1

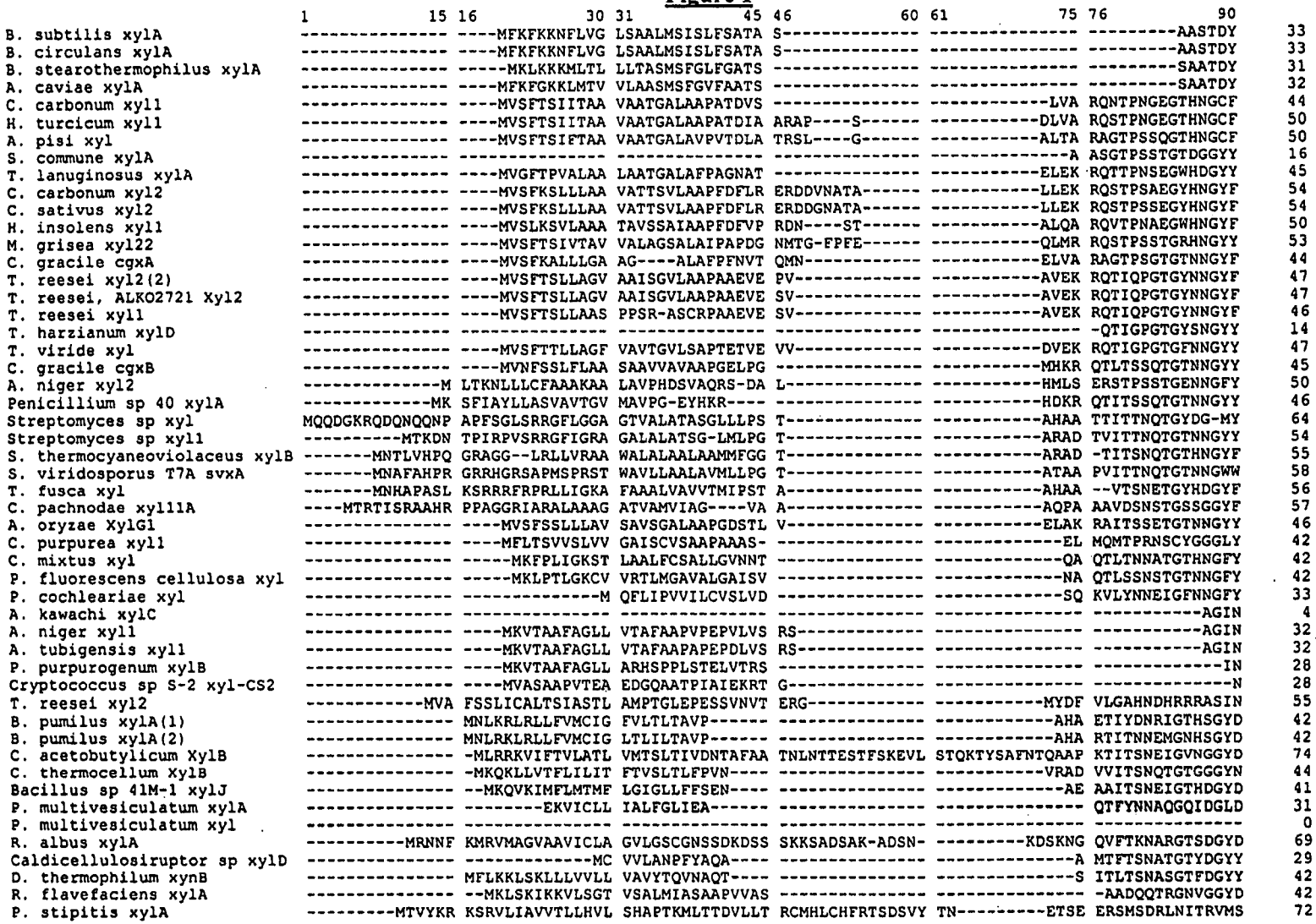
1 15 16 30 31 45 46 60 61 75 76 90
B. subtilis xylA
B. circulans xylA
B. stearothermophilus xylA
A. caviae xylA
C. carbonum xyl1
H. turcicum xyl1
A. pisi xyl
S. commune xylA
T. lanuginosus xylA
C. carbonum xyl2
C. sativus xyl2
H. insolens xyl1
M. grisea xyl22
C. gracile cgxA
T. reesei xyl2(2)
T. reesei, ALKO2721 Xyl2
T. reesei xyl1
T. harzianum xylD
T. viride xyl
C. gracile cgxB
A. niger xyl2
Penicillium sp 40 xylA
Streptomyces sp xyl
Streptomyces sp xyl1
S. thermocyaneoviolaceus xylB
S. viridosporus T7A svxA
T. fusca xyl
C. pachnodae xyl11A
A. oryzae XylG1
C. purpurea xyl1
C. mixtus xyl
P. fluorescens cellulosa xyl
P. cochleariae xyl
A. kawachi xylC
A. niger xyl1
A. tubigensis xyl1
P. purpurogenum xylB
Cryptococcus sp S-2 xyl-CS2
T. reesei xyl2
B. pumilus xylA(1)
B. pumilus xylA(2)
C. acetobutylicum XylB
C. thermocellum XylB
Bacillus sp 41M-1 xylJ
P. multivesiculatum xylA
P. multivesiculatum xyl
R. albus xylA
Caldicellulosiruptor sp xylD
D. thermophilum xynB
R. flavefaciens xylA
P. stipitis xylA

Figure 1 continued

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                                91        105 106       120 121       135 136       150 151       165 166       180
                                WQNWTDGGGIVNAVN GSGGNYSVNWS--NT GNFVVGKGWTTG---  --SPFRTINYNAGVW APNGNG--YLTLYGW TRS-PLIEYVVDSW 113
B. subtilis xylA                WQNWTDGGGIVNAVN GSGGNYSVNWS--NT GNFVVGKGWTTG---  --SPFRTINYNAGVW APNGNG--YLTLYGW TRS-PLIEYVVDSW 113
B. circulans xylA               WQNWTDGGGIVNAVN GSGGNYSVNWS--NT GNFVVGKGWTVG---  --SPNRVINYNAGIW EPSGNG--YLTLYGW TRN-ALIEYVVDSW 111
B. stearothermophilus xylA      WQYWTDGGGMVNAVN GPGGNYSVTWQ--NT GNFVVGKGWTVG---  --TPNRVVNYNAGVF APSGNG--YLTFYGW TRN-ALIEYVVDSW 112
A. caviae xylA                  WQNWTDGGGTVNAVN GSGGNYSVSWQ--NT GNFVVGKGWTVG---  --TPNRVVNYNAGVF APSGNG--YLAVYGW TRN-PLVEYVVENF 122
C. carbonum xyl1                WSWWSDGGARATYTN GAGGSYSVSWG--SG GNLVGGKGWNP----  ---GTARTITYSGTY NYNGNS--YLAIYGW TRN-PLVEYVVENF 128
H. turcicum xyl1                WSWWSDGGARATYTN GAGGSYSVSWG--TG GNLVGGKGWNP----  ---GTARTITYSGQY NPNGNS--YLAIYGW TRN-PLVEYVVENF 128
A. pisi xyl                     YSWWTDGGAQATYTN GAGGSYSVNWK--TG GNLVGGKGWNP----  ---GAARTITYSGTY SPSGNS--YLAVYGW TRN-PLIEYVVENF 128
commune xylA                    YSWWTDGAGDATYQN NGGGSYTLTWSG-NN GNLVGGKGWNP----  --GAASRSISYSGTY QPNGNS--YLSVYGW TRS-SLIEYIVESY 96
S. lanuginosus xylA             YSWWSDGGAQATYTN LEGGTYEISWG--DG GNLVGGKGWNP----  --GLNARAIHFEGVY QPNGNS--YLAVYGW TRN-PLVEYVVENF 124
T. carbonum xyl2                YSWWTDGGGSAQYTM GEGSRYSVTWR--NT GNFVGGKGWNP----  ---GSGRVINYGGAF NPQGNG--YLAVYGW TRN-PLVEYVVIESY 132
C. carbonum xyl2                YSWWTDGGGSAQYTN GEGSRYSVTWR--NT GNFVGGKGWNP----  ---GTGRVINYGGAF NPQGNG--YLAVYGW TRN-PLVEYVVIESY 132
C. sativus xyl2                 YSWWSDGGGQVQYTN LEGSRYQVRWR--NT GNFVGGKGWNP----  ---GTGRTINYGGYF NPQGNG--YLAVYGW TRN-PLVEYVIESY 128
H. insolens xyl1                YSWWTDGASPVQYQN GNGGSYSVQWQ--SG GNFVGGKGWMP----  ---GGSKSITYSGTF NPVNNGNAYLCIYGW TQN-PLVEYYILENY 133
M. grisea xyl22                 YSFWTDGGTVNYQN GAGGSYSVQWQ--NC GNFVGGKGWNP----  ---GAARTINFSGTF SPQGNG--YLAIYGW TQN-PLVEYYIVESF 122
C. gracile cgxA                 HSYWNDGHGGVTYTN GPGGQFSVNWS--NS GNFVGGKGWQP----  --GTKNKVINFSGSY NPNGNS--YLSVYGW SRN-PLIEYYIVENF 126
T. reesei xyl2(2)               YSYWNDGHGGVTYTN GPGGQFSVNWS--NS GNFVGGKGWQP----  --GTKNKVINFSGSY NPNGNS--YLSVYGW SRN-PLIEYYIVENF 126
T. reesei, ALKO2721 Xyl2        YSYWNDGHGGVTYTN GPGGQFSVNWS--NS GNFVGGKGWQP----  --GTKNKVINFSGSY NPNGNS--YLSVYGW SRN-PLIEYYIVENF 125
T. reesei xyl1                  YSYWNDGHGGVTYTN GPGGQFSVNWS--NS GNFVGGKGWQP----  --GTKNKVINFSGSY NPNGNS--YLSIYGW SRN-PLIEYYIVENF 93
T. harzianum xylD               YSYWNDGHAGVTYTN GGGGSFTVNWS--NS GNFVAGKGWQP----  --GTKNKVINFSGSY NPNGNS--YLSIYGW SRN-PLIEYYIVENF 126
T. viride xyl                   YSYWNDGHSGVTYTN GAGGSFSVNWA--NS GNFVGGKGWNP----  --GSSSRVINFSGSY NPNGNS--YLSVYGW SKN-PLIEYYIVENF 123
C. gracile cgxB                 YSFWTDGQGNVQYTN EAGGQYSVTWS--GN GNWVGGKGWNP----  --GS-ARTINYTANY NPNGNS--YLAVYGW TRN-PLIEYYVVENF 123
A. niger xyl2                   YSFWTDGGGDVTYTN GDAGAYTVEWS--NV GNFVGGKGWNP----  ---GSAQDITYSGTF TPSGNG--YLSVYGW TTD-PLIEYYIVESY 128
Penicillium sp 40 xylA          YSFWTNGGGTVQYTN GAAGEYSVTWE--NC GDFTSGKGWST----  ---GSARDITFEGTF NPSGNA--YLAVYGW TTS-PLVEYYILEDY 124
Streptomyces sp xyl             YSFWTDGGGSVSMTL NGGGSYSTQWT--NC GNFVAGKGWGN----  ---GGRRTVRYSGYF NPSGNG--YGCLYGW TSN-PLVEYYIVDNW 142
Streptomyces sp xyl1            YSFWTDGGGSVSMNL ASGGSYGTSWT--NC GNFVAGKGWAN----  ---GARRTVNYSGSF NPSGNA--YLTLYGW TAN-PLVEYYIVDNW 132
S. thermocyaneoviolaceus xyl1B  YSFWTDAPGTVTMNT GAGGNYSTQWS--NT GNFVAGKGWAT----  ---GGRRTVTYSGTF NPSGNA--YLALYGW SQN-PLVEYYIVDNW 133
S. viridosporus T7A svxA        YSFWTDAQGTVSMDL GSGGTYSTQWR--NT GNFVAGKGWST----  ---GGRKTVNYSGTF NPSGNA--YLTLYGW TTG-PLIEYYIVDNW 136
T. fusca xyl                    YSFWTDAPGTVSMEL GPGGNYSTSWR--NT GNFVAGKGWAT----  ---GGRRTVTYSASF NPSGNA--YLTLYGW TRN-PLVEYYIVESW 134
C. pachnodae xyl11A             YSFWTDAPGTVSMNL GSGGNYSTSWS--NT GNFVAGKGWST----  ---GSARTISYSGTF NPSGNA--YLAVYGW SHD-PLVEYYIVDSW 135
A. oryzae Xyl61                 YSFWTNGGGDVEYTN GNGGQYSVKWT--NC DNFVAGKGWNP----  ---GSAKTVTYSGEW ESNSNS--YVSLYGW TQN-PLVEYYIVDKY 124
C. purpurea xyl1                SSYWADYG-NTRYSC GAGGHYDLSWG--NG GNVVAGRGWKP----  ---ASPRAVTYSGSW QCNGNC--YLSVYGW TIN-PLVEYYIVENY 119
C. mixtus xyl                   YTFWKDSG-DASMGL QAGGRYTSQWSN-GT NNWVGGKGWNP----  --GG-PKVVTYSGSY NVDNSQNSYLALYGW TRS-PLIEYYVIESY 122
P. fluorescens cellulosa xyl    YTFWKDSG-DASMTL LSGGRYQSSWGN-ST NNWVGGKGWNP----  --GNNSRVISYSGSY GVDSSQNSYLALYGW TRS-PLIEYYVIESY 123
P. cochleariae xyl              YAFWKDSG-SATFTL ESGGRYAGNWTT-ST NNWVGGKGWNP----  --GNSWRTVNYSGYY GINEYANSYLSLYGW TTN-PLIEYYVVESY 114
A. kawachi xylC                 YVQNYNGNLGDFTYD ESAGTFSMYWEDGVS SDFVVGLGWTT----  --GS-SNAITYSAEY SASGSS-SYLAVYGW VNY-PQAEYYIVEDY 85
A. niger xyl1                   YVQNYNGNLGDFTYD ESAGTFSMYWEDGVS SDFVVGLGWTT----  --GS-SKAITYSAEY SASGSS-SYLAVYGW VNY-PQAEYYIVEDY 113
A. tubigensis xyl1              YVQNYNGNLGDFTYD ESAGTFSMYWEDGVS SDFVVGLGWTT----  --G--SSTITYSAEY SASGSA-SYLAVYGW VNY-PQAEYYIVEDY 112
P. purpurogenum xylB            YVQNYNGNLGAFSYN EGAGTFSMYWQQGVS NDFVVGLGRST----  --GS-SNPITYSASY SASGG--SYLAVYGW VNS-PQAEYVVEAY 108
Cryptococcus sp S-2 xyl-CS2     YVQNYNGNVANFEYS QYDGTFSVNWNG--N TDFVCGLGWTV----  --GT-GRTITYSGSY NPGYSG-SYQAIYGW TGQGSLSEYYVIDNY 108
T. reesei xyl2                  YDQNYQT-GGQVSYS PSNTGFSVNWNT--Q DDFVVGVGWTT----  --GS-SAPINFGGSF SVNSGT-GLLSVYGW STN-PLVEYYIMEDN 133
B. pumilus xylA(1)              FELWKDYG-NTSMTL NNGGAFSASWNNIGN ALFRKGKKFDSTKTH HQ-LGNISINYNAAF NPGGNS--YLCVIGW TQS-PLAEYIVESW 127
B. pumilus xylA(2)              YELWKDYG-NTSMTL NNGGAFSAGWNNIGN ALFRKGKKFDSTRTH HQ-LGNISINVNASF NPGGNS--YLCVYGW TQS-PLAEYIVDSW 127
C. acetobutylicum XylB          YELWKDYG-NTSMTL KNGGAFSCQWSNIGN ALFRKGKKFNDTQTY KQ-LGNISVNYDCNY QPYGNS--YLCVIGW TSS-PLVEYIVDSW 159
C. thermocellum XylB            FEYWKDTG-NGTMVL KDGGAFSCEWSNINN ILFRKGFKYDETKTH DQ-LGYITVTYSCNY QPNGNS--YLGVYGW TSN-PLVEYIIESW 129
Bacillus sp 41M-1 xylJ          YEFWKDSGGSGSGMTL NSGGTFSAQWSNVNN ILFRKGKKFDETQTH QQ-IGNMSINYGATY NPNGNS--YLTVIGW TVD-PLVEFYIVDSW 127
P. multivesiculatum xylA        YELWKDTG-TTSMTL LGGGKFSCWSNINN CLFRIGKKWNCQYEW WE-LGTVLVNYDVDY NPNGNS--YLCIYGW TRN-PLVEYYIESW 116
P. multivesiculatum xyl         ----------MTL LGGGKFSCNWSIGN ALFRIGKKWDCTKTW QQ-LGTISVATNVDY RPNGNS--YMCVYGW TRS-PLIEYYIVDSW 74
R. albus xylA                   YELWKDKG-DTEMTI NEGGTFSCKWSNINN ALFRRGKKFDCTKTY KE-LGNISVKYGVDY QPDGNS--YMCVYGW TID-PLVEFYIESW 154
Caldicellulosiruptor sp xylD    YELWKDTG-NTTMTV DTGGRFSCQWSNINN ALFNTGKKFS--TAW NQ-LGTVKITYSATY NPNGNS--YLCIYGW SRN-PLVEFYIESW 112
D. thermophilum xynB            YELWKDTG-NTTMTV YTQGRFSCQWSNINN ALFRTGKKYN--QNW QS-LGTIRITYSATY NPNGNS--YLCIYGW STN-PLVEFYIESW 125
R. flavefaciens xylA            YEMWNQNGQGQASMN PGAGSFTCSWSNIEN FLARMGKNYDSQKKN YKAFGNIVLTVDVEY TPRGNS--YMCVCGW TRN-PLMEYYIVEGW 129
P. stipitis xylA                YDRWTDLVGELEVRE LKHVMSHRTYSLCDL SCSTVLDSNS------  ---MFSLGKGWQAIS SRQGVG---ATVYGW TRSPLIEYYVVDSW 151
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Figure 1 continued

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                                181       195 196       210 211       225 226       240 241       255 256       270
B. subtilis xylA                GTYRPTGTYKG---- TVKSDGGTYDIYTTT RVNAPSIDGDRTTFT QYWSVRQSKRPTG-- --------SNATITF SNHVNAWKSHGMNLG 189
B. circulans xylA               GTYRPTGTYKG---- TVKSDGGTYDIYTTT RVNAPSIDGDRTTFT QYWSVRQSKRPTG-- --------SNATITF TNHVNAWKSHGMNLG 189
B. stearothermophilus xylA      GTYRATGNYESG--- TVNSDGGTYDIYTTM RVNAPSIDG-TQTFQ QFWSVRQSKRPTG-- --------SNVSITF SNHVNAWRSKGMNLG 187
A. caviae xylA                  GTYRPTGTYKG---- TVNSDGGTYDIYTTM RVNAPSIDG-TQTFP QYWSVRQSKRPTG-- --------VNSTITF SNHVNAWPSKGMYLG 187
C. carbonum xyl1                GTYDPSSQS--QNKG TVTSDGSSYKIAQST RTNQPSIDG-TRTFQ QYWSVRQNKRSSG-- ---------SVNM KTHFDAWASKGMNLG 196
H. turcicum xyl1                GTYDPSSQA--QNKG TVTSDGSSYKIAQST RTNQPSIDG-TRTFQ QYWSVRQNKRSSG-- ---------SVNM KTHFDAWASKGMNLG 202
A. pisi xyl                     GTYDPSSQA--TVKG SVTADGSSYKIAQTQ RTNQPSIDG-TQTFQ QYWSVRQNKRSSG-- ---------SVNM KTHFDAWAAKGMKLG 202
S. commune xylA                 GSYDPSSAA--SHKG SVTCNGATYDILSTW RVNAPSIDG-TQTFE QFWSVRNPKKAPGG- S--------ISGTVDV QCHFDAWKGLGMNLG 175
T. lanuginosus xylA             GTYDPSSGA--TDLG TVECDGSIYRLGKTT RVNAPSIDG-TQTFD QYWSVRQDKRTSG-- ---------TVQT GCHFDAWARAGLNVN 198
C. carbonum xyl2                GTYNPSSGA--QIKG SFQTDGGTYNVAVST RVNQPSIDG-TRTFQ QYWSVRTQKRVGG-- ---------SVNM QNHFNAWSRYGLNLG 206
C. sativus xyl2                 GTYNPSSGA--QVKG SFQTDGGTYNVAVST RVNQPSIDG-TRTFQ QYWSVRQQKRVGG-- ---------SVNM QNHFNAWSRYGLNLG 206
H. insolens xyl1                GTYNPGSQA--QYKG TFYTDGDQYDIFVST RVNQPSIDG-TRTFQ QYWSIRKNKRVGG-- ---------SVNM QNHFNAWQQHGMPLG 202
M. grisea xyl22                 GEYNPGNSA--QSRG TLQAAGGTYTLHEST RVNQPSIEG-TRTFQ QYWAIRQQKRNSG-- ---------TVNT GEFFQAWERAGMRMG 207
C. gracile cgxA                 GTYDPSSQA--SKFG TIQQDGSTYTIAKTT RVNQPSIEG-TSTFD QYWSVRQNHRSSG-- ---------SVNV AAHFNAWAQAGLKLG 196
T. reesei xyl2(2)               GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- ---------SVNT ANHFNAWAQQGLTLG 200
T. reesei, ALKO2721 Xyl2        GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- ---------SVNT ANHFNAWAQQGLTLG 200
T. reesei xyl1                  GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- ---------SVNT ANHFNAWAQQGLTLG 199
T. harzianum xylD               GTYNPSTGA--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHRSSG-- ---------SVNT ANHFNAWASHGLTLG 167
T. viride xyl                   GTYNPSTGT--TKLG EVTSDGSVYDIYRTQ RVNQPSIIG-TATFY QYWSVRRNHAPAA-- ---------RSRL RTTSNAWRNLGLTLG 200
C. gracile cgxB                 GTYNPSTGA--TRLG SVTTDGSCYDIYRTQ RVNQPSIEG-TSTFY QFWSVRQNKRSGG-- ---------SVNM AAHFNAWAAAGLQLG 197
A. niger xyl2                   GDYNPGSGG--TYKG TVTSDGSVYDIYTAT RTNAASIQG-TATFT QYWSVRQNKRVG---- ---------GTVTT SNHFNAWAKLGMNLG 202
Penicillium sp 40 xylA          GDYNPGNSM--TYKG TVTSDGSVYDIYEHQ QVNQPSISG-TATFN QYWSIRQNTRSS---- ---------GTVTT ANHFNAWAKLGMNLG 198
Streptomyces sp xyl             GSYRPTG----EYRG TVYSDGGTYDIYKTT RVNAPSVEG-TRTFD QYWSVRQSKVIGS-- ---------GTITT GNHFDAWARAGMNLG 215
Streptomyces sp xyl1            GTYRPTG----TYKG TVTSDGGTYDVYQTT RVNAPSVEG-TKTFN QYWSVRQSKRTG---- ---------GSITA GNHFDAWARYGMPLG 204
S. thermocyaneoviolaceus xylB   GTYRPTG----TYKG TVYSDGGTYDIYMTT RVNAPSIEG-TKTFN QYWSVRQNKRTG---- ---------GTITT GNHFDAWAAHGMPLG 205
S. viridosporus T7A svxA        GTYRPTG----KYKG TVTSDGGTYDIYKTT RVNAPSIEG-TKTFD QYWSVRQSKRTG---- ---------GTITS GNHFDAWARNGMNLG 208
T. fusca xyl                    GTYRPTG----TYMG TVTTDGGTYDIYKTT RVNAPSIEG-TRTFD QYWSVRQSKRTS---- ---------GTITA GNHFDAWARHGMHLG 206
C. pachnodae xyl11A             GTYRPTG----TFMG TVNSDGGTYDIYKTT RTNAPSIEG-TATFT QYWSVRQSKRVG---- ---------GTITT ANHFNAWASHGMNLG 207
A. oryzae XylG1                 GDYDPSTGA--TELG TVESDGGTYKIYKTT RENAPSIEG-TSTFN QYWSVRQSGRVG---- ---------GTITA QNHFDAWANVGLQLG 198
C. purpurea xyl1                GNYNPSAGA--QRRG QVTADGSIYDIYIST QHNQPSILG-TNTFH QYWSIRRNKRVGG-- ---------TVST GVHFNAWRSLGMPLG 193
C. mixtus xyl                   GSYNPASCSGGTDYG SFQSDGATYNVRRCQ RVQQPSIDG-TQTFY QYFSVRSPKKGFG-- Q--------ISGTITT ANHFNFWASKGLNLG 202
P. fluorescens cellulosa xyl    GSYNPASCSGGTDYG SFQSDGATYNVRRCQ RVNQPSIDG-TQTFY QYFSVRNPKKGFG-- N--------ISGTITF ANHVNFWASKGLNLG 203
P. cochleariae xyl              GSYSPLNCPGGTDEG SFTSGGATYQVRKCR RTNAPSIIG-TQSFD QYFSVRTPKKGFG-- Q--------VSGSVNF ADHVQYWQEMGKLPLG 194
A. kawachi xylC                 GDYNPCSS--ATSLG TVYSDGSTYQVCTDT RTNEPSITG-TSTFT QYFSVRESTRTSG-- ---------TVTV ANHFNFWAQHGFGNS 159
A. niger xyl1                   GDYNPCSS--ATSLG TVYSDGSTYQVCTDT RTNEPSITG-TSTFT QYFSVRESTRTSG-- ---------TVTV ANHFNFWAQHGFGNS 187
A. tubigensis xyl1              GDYNPCSS--ATSLG TVYSDGSTYQVCTDT RTNEPSITG-TSTFT QYFSVRESTRTSG-- ---------TVTV ANHFNFWAHHGFGNS 186
P. purpurogenum xylB            GNYNPCSSGSATNLG TVSSDGGTYQVCTDT RVNQPSITG-TSTFT QFFSVRQGSRTSG-- ---------TVTI ANHFNFWANDGFGNS 184
Cryptococcus sp S-2 xyl-CS2     GGYNPCTGSGVTQLG SLYSDGSSYQVCTHT QYNQPSIVG-TTTFP QYFSVRQNKRSSG-- ---------SVNM QNHFNYWAQHGFPNR 184
T. reesei xyl2                  -HNYPAQG---TVKG TVTSDGATYTIWENT RVNEPSIQG-TATFN QYISVRNSPRTSG-- ---------TVTV QNHFNAWASLGLHLG 205
B. pumilus xylA(1)              GTYRPTG----TYKG SFYADGGTYDIYETL RVNQPSIIG-DATFK QYWSVRQTKRTS---- ---------GTASV SEHFKKWESLGMPMG 199
B. pumilus xylA(2)              GTYRPTG----AYKG SFYADGGTYDIYETT RVNQPSIIG-IATFK QYWSVRQTKRTS---- ---------GTVSV SAHFRKWESLGMPMG 199
C. acetobutylicum XylB          GSWRPPGG---TSKG TITVDGGIYDIYETT RINQPSIQG-NTTFK QYWSVRRTKRTS---- ---------GTISV SKHFAAWESKGMPLG 232
C. thermocellum XylB            GTWRPPGA---TPKG TITVDGGTYEIYETT RVNQPSIKG-TATFQ QYWSVRTSKRTS---- ---------GTISV TEHFKAWERLGMKMG 202
Bacillus sp 41M-1 xylJ          GTWRPPGG---TPKG TINVDGGTYQIYETT RVNQPSIKG-TATFQ QYWSVRTSKRTS---- ---------GTISV SEHFRAWESLGMNMG 200
P. multivesiculatum xylA        GSWRPPGGS--P-MN TMYVDGQYDVYVTD RINQPSIDG-NTNFK QYWSVRTQKKT----- ---------RGTVHV NHHFYNWQEMGLKVG 189
P. multivesiculatum xyl         GSWRPPGSN--S-MG TINVDGGTYDIYVTD RINQPSIDG-TTTFK QFWSVRTQKKT----- ---------SGVISV SKHFEAWTSKGLNLG 147
R. albus xylA                   GSWRPPGAA--ESLG TVTVDGGTYDIYKTT RYEQPSIDG-TKTFD QYWSVRQDKPTGDGT K--------IEGTISI SKHFDAWEQVGLTLG 234
Caldicellulosiruptor sp xylD    GSWRPPG-A--TSLG QVTIDGGTYDIYRTT RVNQPSIVG-TATFD QYWSVRTSKRTS--- ---------GTVTV TDHFKAWAAKGLNLG 185
D. thermophilum xynB            GNWRPPG-A--TSLG QVTIDGGTYDIYRTT RVNQPSIVG-TATFD QYWSVRTSKRTS--- ---------GTVTV TDHFRAWANRGLNLG 198
R. flavefaciens xylA            GDWRPPGND-GEVKG TVSANGNTYDIRKTM RVNQPSLDG-TATFP QYWSVRQTSGSANNQ TN-----YMKGTIDV TKHFDAWSAAGLDMS 212
P. stipitis xylA                GSYHPSNTIT-GTFV TVKCDGGTYDIYTAV RVNAPSIEG--TTFT QYWSVRQSATIQLAV IKPLTLQNATITFTF SNHFDAWKTMTLEAT 238
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Figure 1 continued

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                                271       285 286       300 301       315 316       330 331       345 346       360
B. subtilis xylA                SNWAYQVMATEGYQS SGSSNVTVW------ --------------- --------------- --------------- --------------- 213
B. circulans xylA               SNWAYQVMATEGYQS SGSSNVTVW------ --------------- --------------- --------------- --------------- 213
B. stearothermophilus xylA      SSWAYQVLATEGYQS SGRSNVTVW------ --------------- --------------- --------------- --------------- 211
A. caviae xylA                  NSWSYQVMATEGYQS SGNANVTVW------ --------------- --------------- --------------- --------------- 211
C. carbonum xyl1                -QHYYQIVATEGYFS TGNAQITVNCP---- --------------- --------------- --------------- --------------- 221
H. turcicum xyl1                -SHYYQIVATEGYFS SGSASITVNCP---- --------------- --------------- --------------- --------------- 227
A. pisi xyl                     -THNYQIVATEGYFS SGSAQITVNCA---- --------------- --------------- --------------- --------------- 227
S. commune xylA                 SEHNYQIVATEGYQS SGTATITVTAS---- --------------- --------------- --------------- --------------- 201
T. lanuginosus xylA             GDHYYQIVATEGYFS SGYARITVADVG--- --------------- --------------- --------------- --------------- 225
C. carbonum xyl2                -QHYYQIVATEGYQS SGSSDIYVQTQ---- --------------- --------------- --------------- --------------- 231
C. sativus xyl2                 -QHYYQIVATEGYQS SGSSDIYVQTQ---- --------------- --------------- --------------- --------------- 231
H. insolens xyl1                -QHYYQVVATEGYQS SGESDIYVQTH---- --------------- --------------- --------------- --------------- 227
M. grisea xyl22                 -NHNYMIVATEGYRS AGNSNINVQTPA--- --------------- --------------- --------------- --------------- 233
C. gracile cgxA                 -SHNYQIVATEGYQS SGSSSITVS------ --------------- --------------- --------------- --------------- 219
T. reesei xyl2(2)               -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- --------------- 223
T. reesei, ALKO2721 Xyl2        -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- --------------- 223
T. reesei xyl1                  -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- --------------- 222
T. harzianum xylD               -TMDYQIVAVEGYFS SGSASITVS------ --------------- --------------- --------------- --------------- 190
T. viride xyl                   -TLDYQIIAVEGYFS SGNANINVS------ --------------- --------------- --------------- --------------- 223
C. gracile cgxB                 -THDYQIVATEGYYS SGSATVNVGASSDGS TGGGSTGGGSTNVSF --------------- --------------- --------------- 241
A. niger xyl2                   THN-YQIVATEGYQS SGSSSITVQ------ --------------- --------------- --------------- --------------- 225
Penicillium sp 40 xylA          SFN-YQIVSTEGYES SGSSTITVS------ --------------- --------------- --------------- --------------- 221
Streptomyces sp xyl             QFQYYMIMATEGYQS SGSSNITVSG----- --------------- --------------- --------------- --------------- 240
Streptomyces sp xyl1            SFNYYMIMATEGYQS SGSSSISVS------ --------------- --------------- --------------- --------------- 228
S. thermocyaneoviolaceus xylB   TFN-YMILATEGYQS SGSSNITVGDSGGDN GGGGG---------- --------------- --------------- --------------- 292
S. viridosporus T7A svxA        NHN-YMIMATEGYQS SGSSTITVSESGSGG GGGGG---------- --------------- --------------- --------------- 286
T. fusca xyl                    THD-YMIMATEGYQS SGSSNVTLGTSGGGN PGGGN---------- --------------- --------------- --------------- 295
C. pachnodae xyl11A             RHD-YQILATEGYQS SGSSNITIGSTSGGG GSGGG---------- --------------- --------------- --------------- 293
A. oryzae XylG1                 THN-YMILATEGYKS SGSATITVE------ --------------- --------------- --------------- --------------- 221
C. purpurea xyl1                -TYDYMIVATEGFRS SGSASITVS------ --------------- --------------- --------------- --------------- 216
C. mixtus xyl                   -NHDYMVLATEGYQS RGSSDITVSEGTGGT TSSSV---------- --------------- --------------- --------------- 290
P. fluorescens cellulosa xyl    -NHNYQVLATEGYQS RGSSDITVSEGTSGG GTSSV---------- --------------- --------------- --------------- 292
P. cochleariae xyl              -THAHQIFATEGYQS SGFADITVS------ --------------- --------------- --------------- --------------- 217
A. kawachi xylC                 DFN-YQVMAVEAWSG AGSASVTIS------ --------------- --------------- --------------- --------------- 182
A. niger xyl1                   DFN-YQVMAVEAWSG AGSASVTISS----- --------------- --------------- --------------- --------------- 211
A. tubigensis xyl1              DFN-YQVVAVEAWSG AGSASVTISS----- --------------- --------------- --------------- --------------- 210
P. purpurogenum xylB            NFN-YQVVAVEAWSG TGTASVTVSA----- --------------- --------------- --------------- --------------- 208
Cryptococcus sp S-2 xyl-CS2     NFN-YQVLAVEGFSG SGNANMKLISG---- --------------- --------------- --------------- --------------- 209
T. reesei xyl2                  QMN-YQVVAVEGWGG SGSASQSVSN----- --------------- --------------- --------------- --------------- 229
B. pumilus xylA(1)              -KMYETALTVEGYRS NGSANVMTNQLMIR- --------------- --------------- --------------- --------------- 227
B. pumilus xylA(2)              -KMYETAFTVEGYQS SGSANVMTNQLFIGN --------------- --------------- --------------- --------------- 228
C. acetobutylicum XylB          -KMHETAFNIEGYQS SGKADVNSMSINIGK --------------- --------------- --------------- --------------- 261
C. thermocellum XylB            -KMYEVALVVEGYQS SGKADVTSMTITVGN APS------------ --------------- --------------- --------------- 277
Bacillus sp 41M-1 xylJ          -NMYEVALTVEGYQS SGSANVYSNTLTIGG Q-------------- --------------- --------------- --------------- 268
P. multivesiculatum xylA        -KVYEASLNIEGYQS AGSATVNKNEVVQT- --------------- --------------- --------------- --------------- 217
P. multivesiculatum xyl         -LMYEASLTIEGYQS SGSATVNQNDVTGG- --------------- --------------- --------------- --------------- 175
R. albus xylA                   -NMYEVALNIEGYQS NGQATIYENELTVDG NY------------- --------------- --------------- --------------- 303
Caldicellulosiruptor sp xylD    -TIDQITLCVEGYQS SGSANITQNTFTIGG SSSGSSNGSNNG--- --------------- --------------- --------------- 274
D. thermophilum xynB            -TIDQITLCVEGYQS SGSANITQNTFSQG- ----SSSGSS----- --------------- --------------- --------------- 273
R. flavefaciens xylA            GTLYEVSLNIEGYRS NGSANVKSVSVTQGG SSDNGGQQQNNDW-- --------------- --------------- --------------- 255
P. stipitis xylA                HSTEGYFSSGITYEQ PHQPHRNTWATSLTS QTKHTARSLPIN--- --------------- --------------- --------------- 328
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 02/03797

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N9/24 C12N9/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, MEDLINE, BIOSIS, SEQUENCE SEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 5 306 633 A (SCHUSTER ERWIN ET AL) 26 April 1994 (1994-04-26) the whole document	7-13,17, 20-23 1-23
X Y	WO 00 39289 A (SOERENSEN JENS FRISBAEK ;DANISCO (DK); SIBBESEN OLE (DK)) 6 July 2000 (2000-07-06) page 19, line 13 page 63, line 25 - line 28; claims 1-7; figure 20; table 14	7-13,17, 20-23 1-23
X	US 5 736 384 A (FUKUNAGA NOBUYUKI ET AL) 7 April 1998 (1998-04-07) column 3 -column 20; claim 3; figures 1-5	7-13, 17-23
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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
16 January 2003	24/01/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Stoyanov, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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